ResGen™
Invitrogen Corporation

Pathways™ 4 Software

User's Guide

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International customers refer to www.invitrogen.com for technical support contact information.

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Book I: Introduction and Overview

Chapter 1: Highlights

1.1 Introduction

As a comprehensive tool for the analysis of microarray data, the Pathways™ 4 software unleashes microarrays' potential for discovery. From image importing through data mining, data analysis is rapid, accurate, and extensible.

This manual describes the process of data analysis in Pathways™ 4. Book I offers a product overview, including program highlights and a tour of the graphical interface. Book II introduces new features exclusive to Pathways™ Universal. Book III describes the flow of data and the image importing process. Book IV addresses some of the core concepts in Pathways™ data analysis, including project organization, data normalization, and filtering tools for large data set reduction. Book V describes the three primary types of data analysis (comparison, profiling, and clustering), and it offers practical examples of each technique.

1.2 Highlights of the Software

The Pathways™ 4 software encompasses a sophisticated and comprehensive set of tools for the analysis of differential gene expression using microarray data. The following highlights are included.

- **Batch importing for rapid, automated importing of multiple microarray images.**
- **Support for multiple image formats, including Tiff, Fuji, and Pathways™ image formats.**
- **Multiple views of each data set and analysis results, including scatter plots, tables, and synthetic images of the microarray.**
- **Statistical analysis of data sets, including unrelated t-tests and ANOVA.**
- **Isolation of genes in large data sets by filtering based on user-specified criteria.**
- **Multiple clustering algorithms, including KMeans, Hierarchical clustering, and SOM.**
- **An embedded browser enabling hyperlinks between a clone and public sites like the National Center for Biotechnology Information's (NCBI's) GenBank® and Unigene databases.**
- **Updating of default data files and plug-ins from the ResGen™ Data Server to ensure that data for each clone is always current (subscription service).**
- **Java architecture for ease of cross-platform use.**
- **Multiple pluggable components, allowing Pathways™ functionality to be extended by ResGen™, third party vendors, or end users.**

1.3 Hardware and Software Requirements

Processor: Pentium II, 400 MHz or better

Memory: 256 MB RAM

Hard drive: Core program 45 MB, including Java runtime environment and two sample images

Operating system: Windows (95, 98, 2000, or NT), Linux, Solaris, Macintosh OSX, or any platform supporting the Java 1.3 runtime environment.

Video: SVGA with 1024 x 768 resolution or better, 256 color palette or better

1.4 Architecture of the Program

The Pathways™ 4 software is written in Java 1.3, allowing significant flexibility in the choice of operating systems, extensibility of the program, and ease of internet data access.

1.5 Pluggable Interfaces

Pathways™ is designed with multiple "pluggable" components that allow the program to be extended at runtime by placing new java jar files in the appropriate Pathways™ distribution directory. Pluggable components include sampling, clustering (core algorithms and visualization), normalization, data analysis, image formats, microarray descriptions and web interconnectivity. New plug-ins can be created by ResGen™, third party software vendors, or end users.

1.6 Compatibility with Previous Versions of Pathways™

Pathways™ 4 represents a significant advance in technology over Pathways™ 2.0. The algorithms for autocentering imported images, sampling, and normalization have been revised and improved. As an improvement on Pathways™ 2, Pathways™ 4 includes the following analysis capabilities.

- **Statistical analysis**
- **Clustering**
- **Multiple modes of visualization**

Furthermore, Pathways™ 4 stores microarray data in sharable files, whereas Pathways™ 2.0 stored data in Microsoft Access databases. These improvements mean that microarray images that were previously imported into Pathways™ 2.0 must be reimported into Pathways™ 4. The improved import process allows rapid reimport of previously imported data.

Pathways™ 4 is fully compatible with all Pathways™ 3 projects and image files.

1.7 Comparison of Pathways™ 4 Universal to Pathways™ 4 GeneFilters®

Pathways™ 4 Universal supports multiple microarray products, while Pathways™ 4 GeneFilters® software is designed specifically for ResGen™ GeneFilters® microarrays. Features exclusive to Pathways™ 4 Universal include the Array Designer and Frameworks, which allow for analysis of other microarray products or previously analyzed array intensities. Refer to Book II for more information about the Array Designer and Frameworks.

Whenever this symbol appears, the text following it refers to features exclusive to Pathways™ 4 Universal.

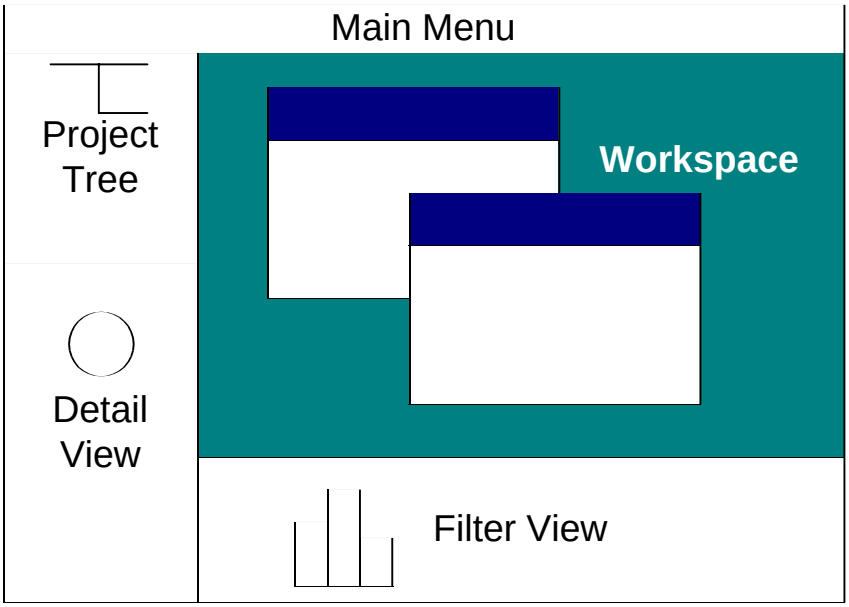
Chapter 2: Overview of the Graphical User Interface

This chapter offers an overview of the Pathways™ Graphical User Interface (GUI). Detailed descriptions of importing, project creation, and data analysis are presented in later chapters.

2.1 Layout of the Graphical User Interface

The Pathways™ GUI has five primary sections.

- **Main Menu**
- **Workspace**
- **Project Tree**
- **Detail View**
- **Filter View**



The main menu contains menu items for all aspects of the Pathways™ program, from file management to data analysis. The workspace contains active analysis windows for the current project. The project tree shows the current project's microarrays. The detail view shows relevant information about the currently selected clone(s) in the active analysis window, including a thumbnail view of the clone in the original experimental image. The filter view displays data filters that are available for the currently active analysis window. Each of these sections is discussed in more detail in the following text.

Each section of the GUI is divided by borders that can be resized to allow more space for a section of the GUI. To change the size of a section:

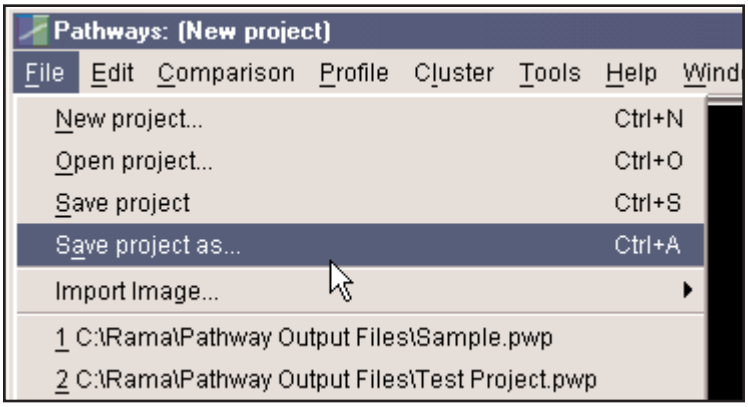
Move the cursor over the border that needs to be moved.

A double-headed arrow appears.

Click and drag the border.

2.2 Menus

The **File** menu commands create and save projects, import images, and exit the program.



New Project: **Opens a new project dialog.**

Open Project: **Opens a dialog box from which a saved project can be loaded.**

Save Project: **Saves the current project.**

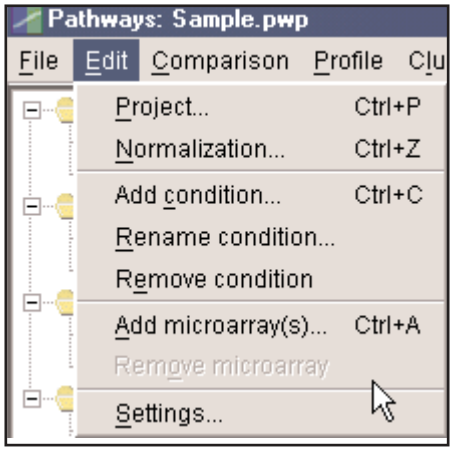
Save Project As: **Saves the Current Project as a new file name.**

Import Image: **Opens a dialog box for importing a new microarray image.**

Recent Projects: **A list of recently opened projects appears here.**

Exit: **Exits the program.**

The **Edit** menu offers a variety of choices for editing the current project and allows general program settings to be adjusted.



Project: Edit project properties such as project and researcher name.

Normalization: Edit the normalization groups and methods for the current project.

Add Condition: Add a condition to the current project.

Rename Condition: Rename the current condition (active only when a condition is selected in the Project Tree).

Remove Condition: Remove the current condition from the project tree (active only when a condition is selected in the Project Tree).

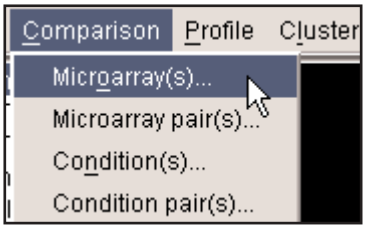
Add Microarray: Add a microarray to the current condition (active only when a condition is selected in the Project Tree).

Remove Microarray: Remove the current microarray (active only when a microarray is selected in the Project Tree).

Settings: General program settings (see Settings section).

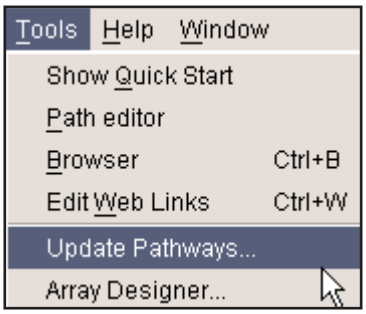
The **Comparison**, **Profile**, and **Cluster** menus represent the core analysis capabilities of Pathways™. Comparison analysis is used to analyze data for an entire set of clones (*e. g.*, determination of upregulation or downregulation of clones over two experiments). Profiling analysis views data as a function of experimental conditions (*e. g.*, determination of the general trending of a subset of genes in a time course study). Clustering analysis generates associations between clones in a data set, automatically.

Each analysis menu has four submenus that represent how microarray data could be grouped for the analysis: **Microarray(s)**, **Microarray Pair(s)**, **Condition(s)**, **Condition Pair(s)**.



These analysis modes and the analysis groupings are discussed in detail in later chapters.

The **Tools** menu offers access to the **Quick Start** palette, **Path editor**, an internet **Browser** window, and a **Web link** editor. **Updating** of Pathways™ data and plug-ins is also launched from the Tools menu. The **Array Designer** is only available in Pathways™ 4 Universal (refer to Chapter 4 for more information on the Array Designer).



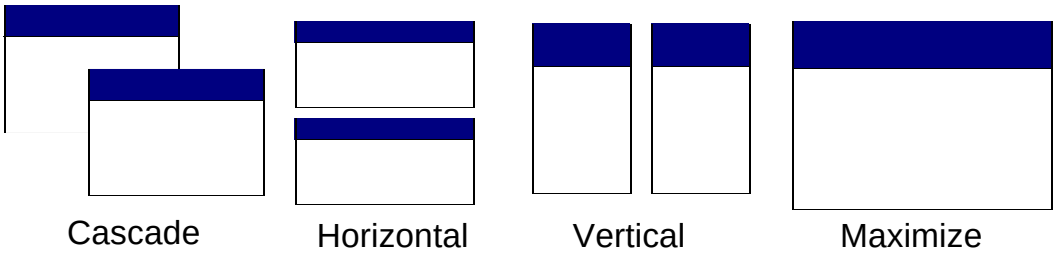
The **Help** menu offers access to help on the Pathways™ program, including general information about the program, and a searchable version of the Pathways™ manual.

The **Windows** menu presents several options for managing the active analysis windows in the workspace. For details, refer to the description of the workspace.

2.3 Workspace

During a Pathways™ analysis session, the Workspace may contain multiple analysis windows. The current analysis window completes the appropriate information in the detail and filter views (the browser, for example, does not use the detail and filter views).

When an analysis window is open, the **Window** menu options on the main screen are active. The four options (**Cascade**, **Maximize**, **Tile Horizontally** and **Tile Vertically**) allow management of multiple windows simultaneously.

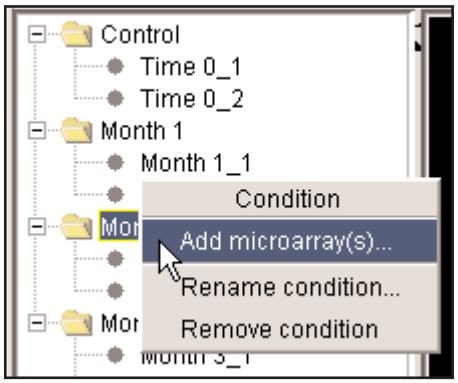


In addition, each current analysis window is displayed in this menu.

An analysis window displays a **title bar** at the top of the window, along with minimize, maximize, and close buttons. The title bar displays the type of analysis. It also gives details of the averaging method (microarray address or clone address, refer to Chapter 7 for more information) used for data analysis.

2.4 Project Tree

The **Project Tree** area displays the current Project as a tree of conditions and their microarrays. Right clicking in the Tree window generates menus that serve as a shortcut to much of the functionality in the **Edit** menu.



Right clicking **outside any conditions** generates a menu that allows adding a condition. Right clicking **on a condition** generates a menu that allows adding a microarray to the condition or renaming or removing the condition. Right clicking **on a microarray** generates a menu that allows removing the microarray from the project. Adding or removing conditions or microarrays to the project generates a prompt to close any active analysis windows that would be affected by the modification.

2.5 Detail View

The detail view area of the GUI shows thumbnail pictures of the analysis window's currently selected clone, along with a table of information on the selected clones. The detail view area starts with web links, then thumbnail(s), and finally an information table (associated meta data).

The detail view area of the GUI may appear different if a framework is used that does not supply geometry.

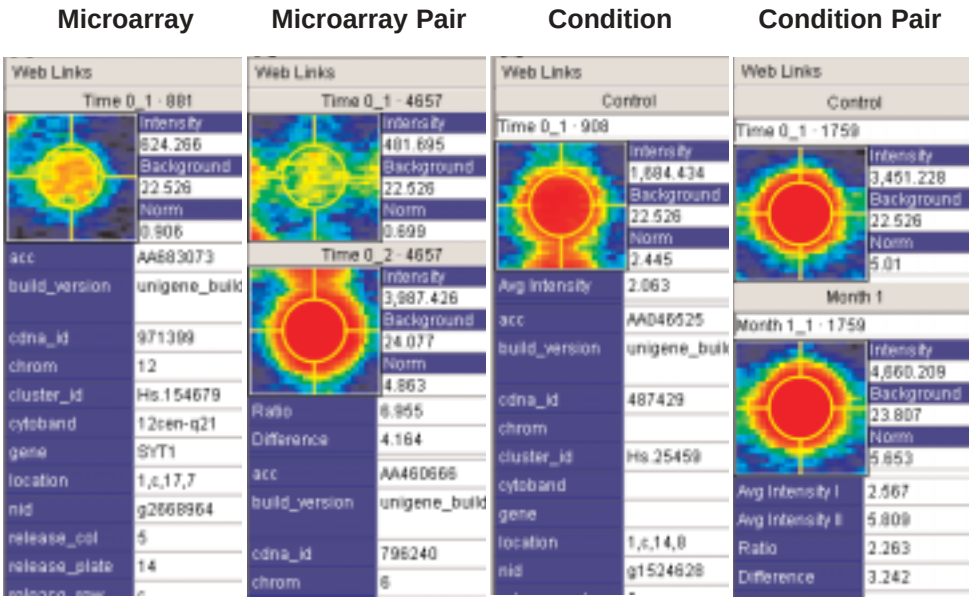
The *Web Links* button at the top of the detail view opens a web browser that connects to Unigene, Genecards, and other web sites that display information related to the clone. These sites can be expanded by either editing links through the Tools menu or through the **pluggable** web interface (refer to the Data Updating chapter).

The forms of the thumbnail section depend on the grouping selected for the analysis.

- **Microarray(s): A thumbnail image is shown for the currently selected clone.**
- **Microarray Pair(s): A thumbnail image is shown of the current clone from each of the two microarrays. The ratio and difference of the normalized intensities are shown in the meta data table below the thumbnails.**
- **Condition(s): A thumbnail for each microarray that contributes to the current point is 'stacked' on the thumbnail view. A spinner control flips through each thumbnail (if this data point does not have any repeats, then the spinner is disabled). The average normalized intensity for the currently selected clone is displayed in the table below the thumbnail.**
- **Condition Pairs(s): Condition pairs are displayed as two 'stacked' condition thumb nails. The average normalized intensity for each condition is listed in the table below, as are the ratio and difference between the pairs. Because each condition can contain clones from different microarrays, a check box next to each condition label indicates the condition from which to derive the meta data in the table.**

The ratio is displayed in a '+ / - ratio' format, meaning that upregulation (B>A) is displayed as positive B divided by A and downregulation (B<A) is displayed as negative A divided by B.

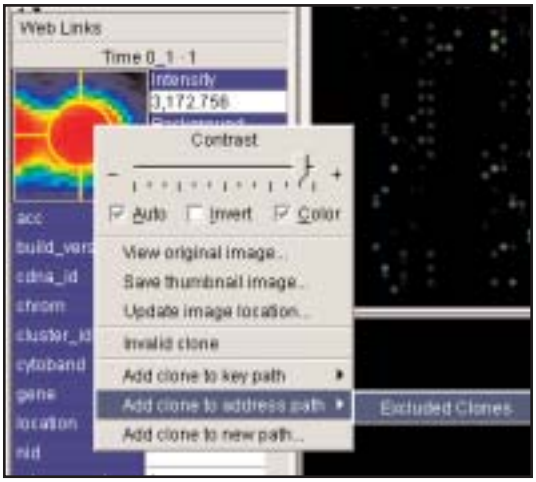
Examples of each detail view are shown below.



The **intensity**, **background**, and **normalized intensity** values are displayed to the right of each thumbnail image. The signal intensity and background intensity represent the sampled intensity values that were measured during the importing process. The normalized intensity is the calculated normalized intensity for this clone in the current project. The background value is the raw intensity, useful when evaluating low intensity points. Genes with intensities at or near the background average could be noise.

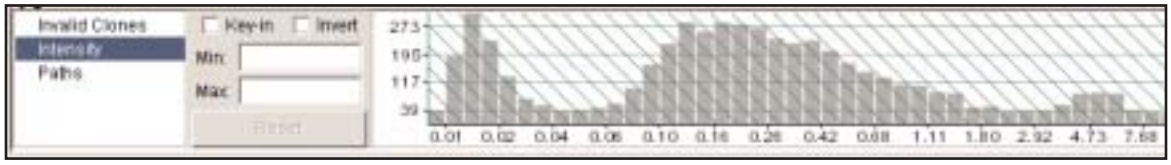
Meta data such as cluster ID, accession number, and cDNA ID is also listed in the detail view. This data can be updated as new information becomes available through the ResGen™ data server (for details, refer to Chapter 14).

Right clicking on the thumbnail image generates a menu with options for contrasting the image (see Contrast Controller, below), viewing the original image, and saving the thumbnail images. Image and data files are stored separately, and if a data file is moved, the location of the image file must be updated. The menu includes an option updating the location of the image. Additionally, options for adding the current clone to an existing or new path or invalidating a particular clone appear in the menu.



2.6 Filter View

The filter view displays available data filters for the current analysis window.



When a filter setting is adjusted, a check mark appears beside the filter name in the filter view. Refer to Chapter 9 for a detailed discussion of filter use.

2.7 Quick Start

The Quick Start palette offers a quick method of accessing core functionality in Pathways™ 4, including the Array Designer (disabled with Pathways™ 4 GeneFilters®).

Pathways Quick-Start
Import a new microarray image
Open an existing project
Open the project wizard to guide you through the creation of a new project
Analyze relative intensity values of a single microarray
Compare two microarrays using intensity ratios and differences
Construct a dynamic list of genes built on your criteria
Design new microarray layouts using the Array Designer tool
Show at start-up
Close

Quick Start appears automatically at startup (unless the option is disabled), or it may be launched at any point from the **Tools→Quick Start** menu. Quick Start reappears after tasks are finished, unless the *Close* button is clicked.

Quick Start performs the following functions.

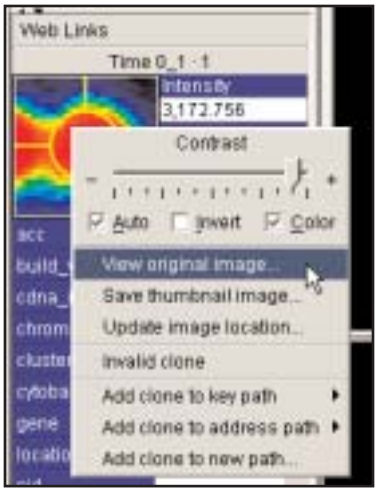
- **Imports images**
- **Opens project files**
- **Launches the new project wizard**
- **Analyzes one or two microarrays**
- **Edits Paths**
- **Launches the Array Designer**

These functions are discussed in detail in this manual.

The Quick Start menu includes the Array Designer in Pathways™ 4 Universal. Refer to Book II for information about using the Array Designer.

2.8 Contrast Controller

A contrast controller is available when an experimental image is displayed in Pathways™. The contrast controller is visible in the left pane of the interactive importing windows. Otherwise, the contrast controller can be accessed through right clicking on any experimental image, such as on the thumbnail in the detail view.



The contrast controller allows enhancement of experimental images.

Contrast slider: generates minimum contrast when the slider is to the left, maximum contrast when the slider is to the right.

Auto: automatically adjusts the brightness levels of the displayed image to the minimum and maximum intensities for the experimental image.

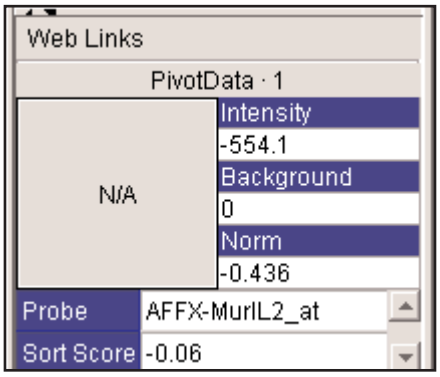
Invert: inverts the image intensities (for example, black on white image is displayed as white on black).

Color: indicates whether the image is displayed in grayscale or color.

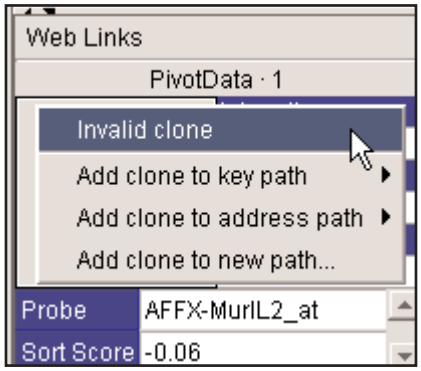
In addition to the standard settings, the contrast controller for the thumbnail has an option to display the entire original image from which the thumbnail is being displayed, to save the thumbnail image, and to update the location of the saved image.

Changing the contrast for the thumbnail image changes the contrast for thumbnails that are displayed for the same microarray. However, this change does not affect the contrast of thumbnails for other microarrays that are being displayed; each image must be contrasted separately.

Thumbnail images are framework-dependent. Some frameworks may not supply the geometry necessary to construct an image of the microarray. In such cases, the thumbnail image will contain the text “N/A,” as shown below.



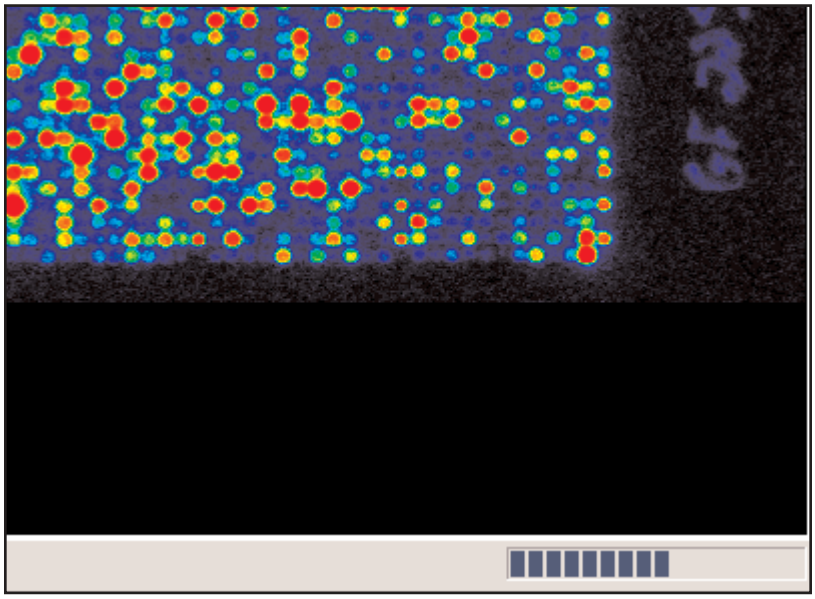
Additionally, right-clicking on the detail view window area generates a different menu.



The options for invalidating a clone and adding a clone to a new or existing path are present, but the thumbnail options are not.

2.9 Progress Bars

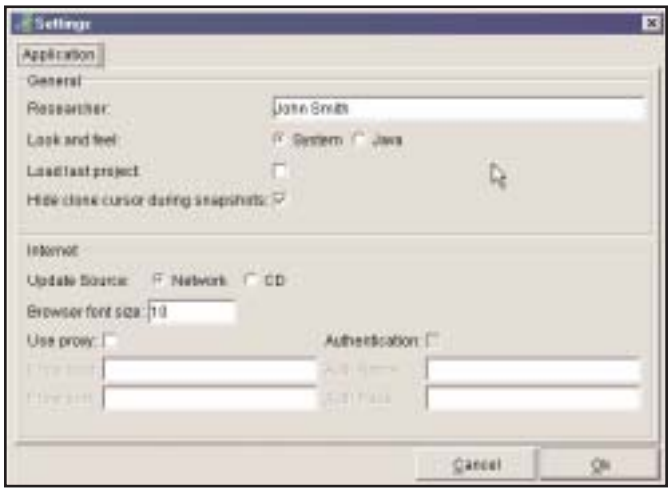
Progress bars are shown in Pathways™ when lengthy operations are performed to indicate the progress of the operation. The progress bar on the lower right of the GUI indicates the progress of a basic operation like loading a file or calculating clone centers in the importing process.



A drawing progress bar appears in the upper left corner of a panel when a time consuming drawing operation is underway, such as drawing a synthetic array or an experimental image.

2.10 General Settings

General settings for Pathways™ 4 are found under the **Edit→Settings** menu.

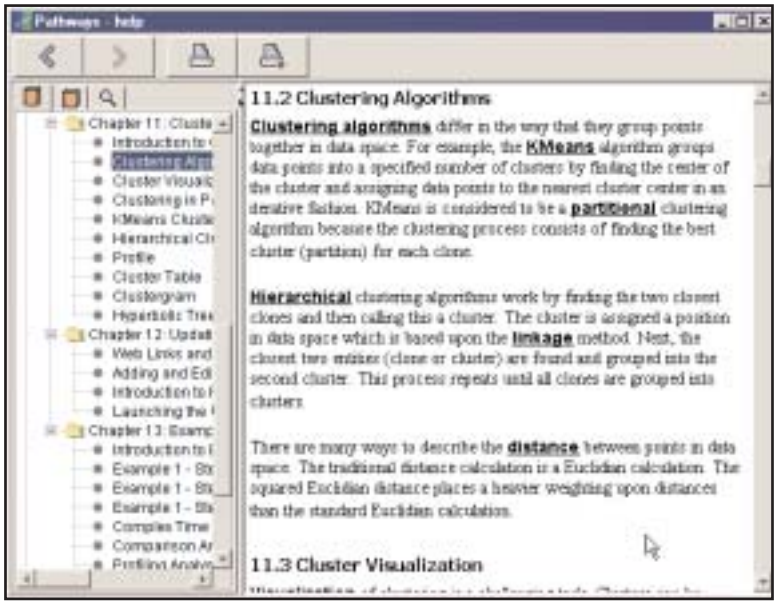


General settings allow entry of a default Researcher name for projects and imports, the preferred look and feel for Pathways™, and whether to load the last project when Pathways™ is started. The **Look and feel** option can be set to **System** (the program would, for example, look like a Windows application when running on a Windows operating system) or **Java** (a characteristic look for Java applications that appear the same on any operating system). The **Load last project** option, when checked, loads the most recent project. The **Hide clone cursor during snapshots** option, when checked, hides the clone cursor while snapshots are being taken.

Internet settings allow specification of the built-in web browser and for online update services. The default options for **Update Source** may be set to either **Network** or **CD**. The default **Browser font size** and **Proxy** settings can be entered. For computers behind a firewall, Pathways™ supports proxies and proxy authentication. To enable proxy support, check the **Use Proxy** checkbox, and enter the host and port in the **Proxy host** and **Proxy port** fields, respectively. Microsoft Proxy™ users must complete the IP address of the proxy and not the windows domain name (for example, \\Proxy is not a valid proxy name). If the proxy requires authentication, check the **Authentication** box, and enter a username and password. The proxy password is stored in an encrypted format in the settings. These proxy settings are used for browser windows (and weblinks) and the update service. Contact the system administrator to determine whether proxy settings are required to access the internet.

2.11 Online Help

The help menu allows access to two submenus: **Help Topics** and **About**. About offers general information on Pathways™ software, including the license key. Help Topics offers online access to the full Pathways™ manual, a glossary of terms, and a keyword search utility.


Pathways - help
11.2 Clustering Algorithms
Clustering algorithms differ in the way that they group points together in data space. For example, the KMeans algorithm groups data points into a specified number of clusters by finding the center of the cluster and assigning data points to the nearest cluster center in an iterative fashion. KMeans is considered to be a partitional clustering algorithm because the clustering process consists of finding the best cluster (partition) for each clone.
Hierarchical clustering algorithms work by finding the two closest clones and then calling this a cluster. The cluster is assigned a position in data space which is based upon the linkage method. Next, the closest two entities (clone or cluster) are found and grouped into the second cluster. This process repeats until all clones are grouped into clusters.
There are many ways to describe the distance between points in data space. The traditional distance calculation is a Euclidian calculation. The squared Euclidian distance places a heavier weighting upon distances than the standard Euclidian calculation.
11.3 Cluster Visualization

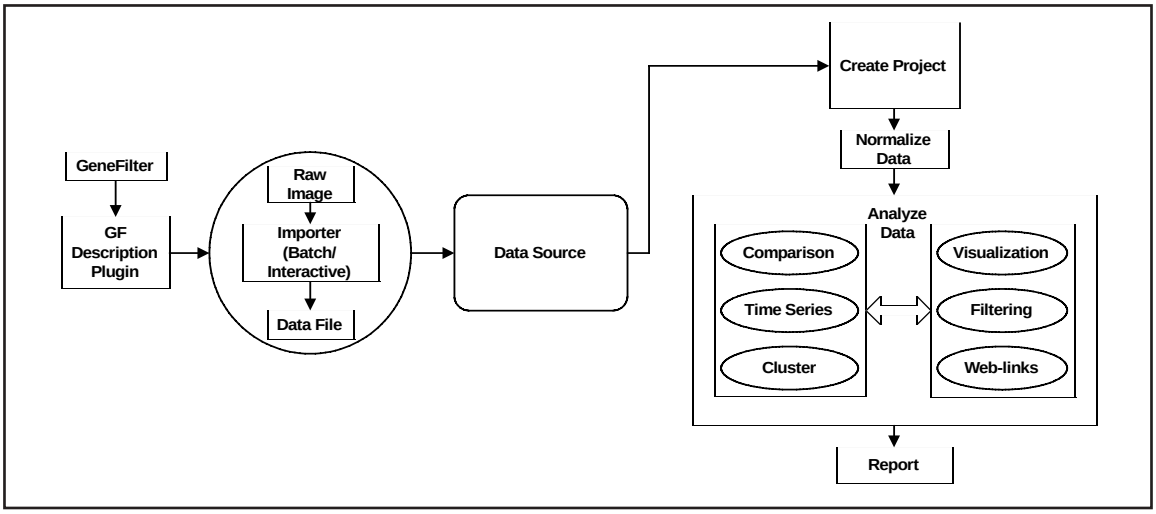
Book II: Universal Concepts

Chapter 3: Frameworks

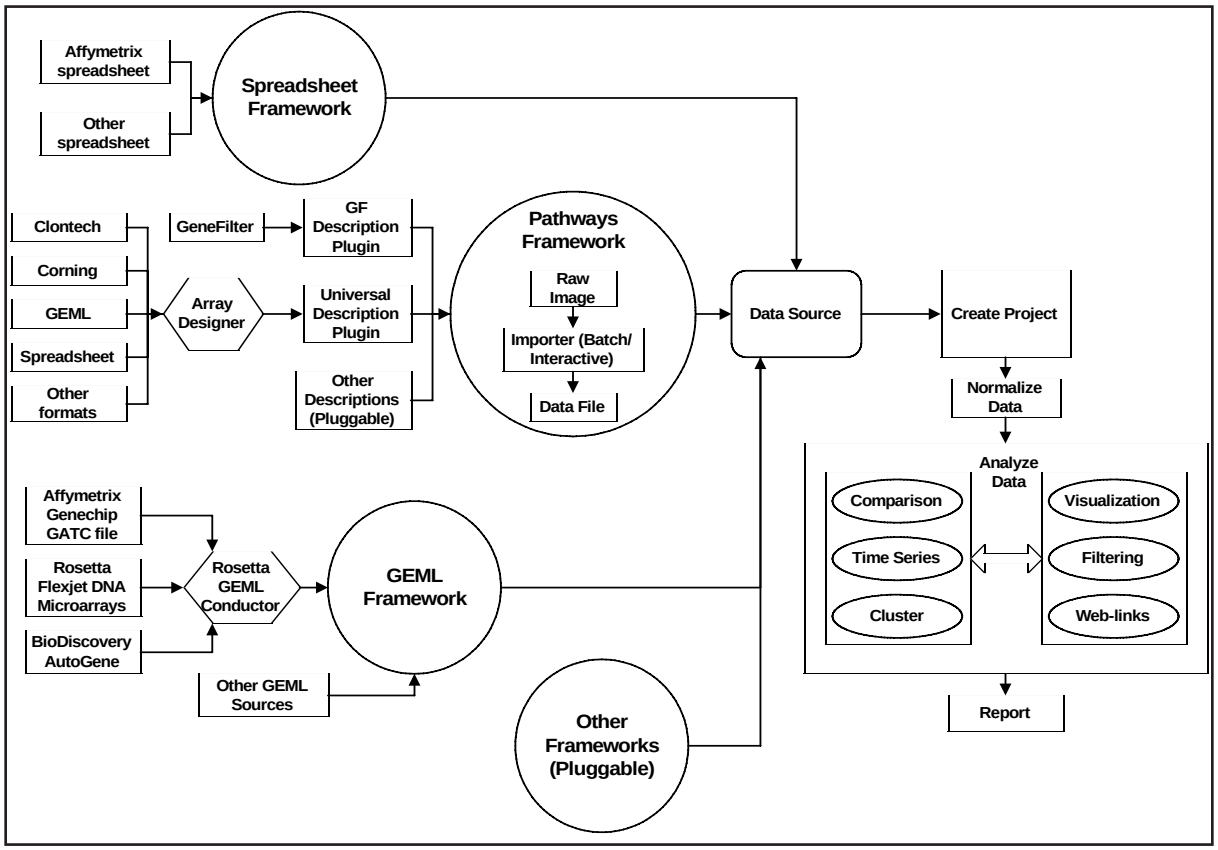
3.1 Introduction to Frameworks

Pathways™ Universal introduces the concept of frameworks. Frameworks are collections of modules that work together to provide a method of importing microarray data from various sources.

Previous versions of Pathways™ came with one framework that limited the importing of microarray data to ResGen™ GeneFilters® image files. ResGen™ GeneFilters® microarrays were imported into the Pathways™ program using the standard Pathways™ framework. Sophisticated image processing techniques then transformed the raw image file into a computational description of the microarray. Pathways™ used this description in its analysis of the microarray.



Pathways™ Universal allows the importing of microarray data from several different sources and formats. In addition to ResGen™ GeneFilters® microarrays, the standard Pathways™ framework now supports other microarray formats through the use of the Array Designer (refer to Chapter 4 for details about the Array Designer). Pathways™ Universal also comes bundled with new framework plug-in modules which can take data from spreadsheet files, Gene Expression Markup Language (GEML™) files, and other formats (*e.g.* databases) through a pluggable interface. Through the use of the framework plug-in modules, Pathways™ can take microarray data from nearly any source and transform that data into a format the program can use for analysis.



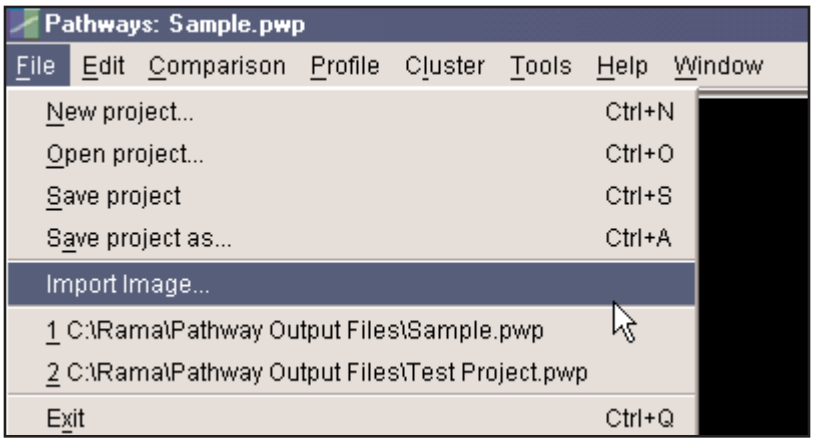
Frameworks provide a means of loading and saving data, but they may serve other functions. Frameworks that use raw image files may provide a different way of importing and viewing images. **The functionality of the framework depends on the type of microarray data being imported.** Because frameworks are plug-in modules, new frameworks for previously unsupported microarray data sources can be developed and integrated into Pathways™.

3.2 Influence of Frameworks

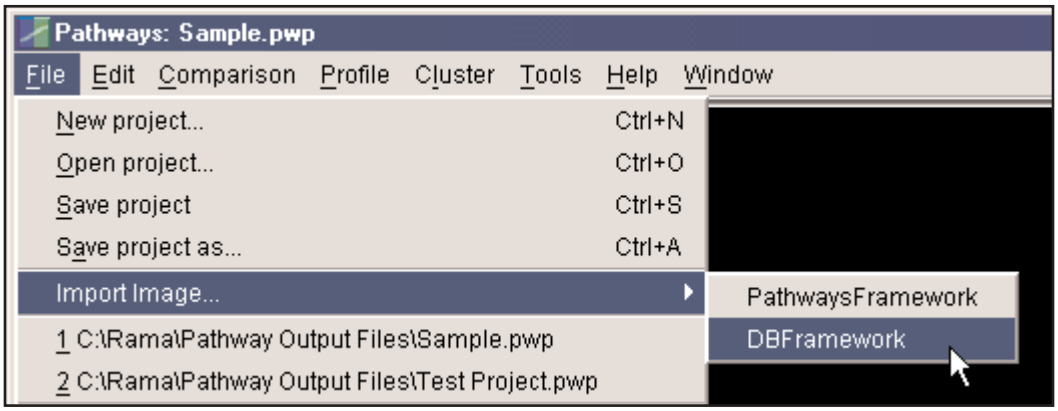
Frameworks influences the **File** menu, where new menu options may be introduced for importing microarray data. Some frameworks obtain their microarray data through a relatively simple process, such as reading the data from a spreadsheet file. For these frameworks, an image import tool is unnecessary. For other frameworks, where the process of converting the original array image into a useful representation is more complicated, the framework must provide an image import tool.

One example of this kind of framework is the standard Pathways™ framework, which transforms an image file into a computational description of a microarray. For Pathways™ to identify the locations of spots on a microarray and then sample their intensities, some user intervention may be required during the process (*e.g.* to verify that the auto-centering algorithm correctly identifies the spots' centers before it samples their intensities).

Since each framework may provide a tool for importing microarray data into Pathways™, the File menu may list multiple import options. The number of import options in the File menu depends on the frameworks available with the Pathways™ installation. For example, when only the standard Pathways™ framework is present, or when other frameworks that do not import other file formats are present, the File menu lists a single import option, **Import Image**.



When an additional framework is present that supports the importing of other file formats, such as a database, the Import Image option becomes a cascading menu with entries for all frameworks that support importing (the database framework is available as a reference implementation in source code format upon request).



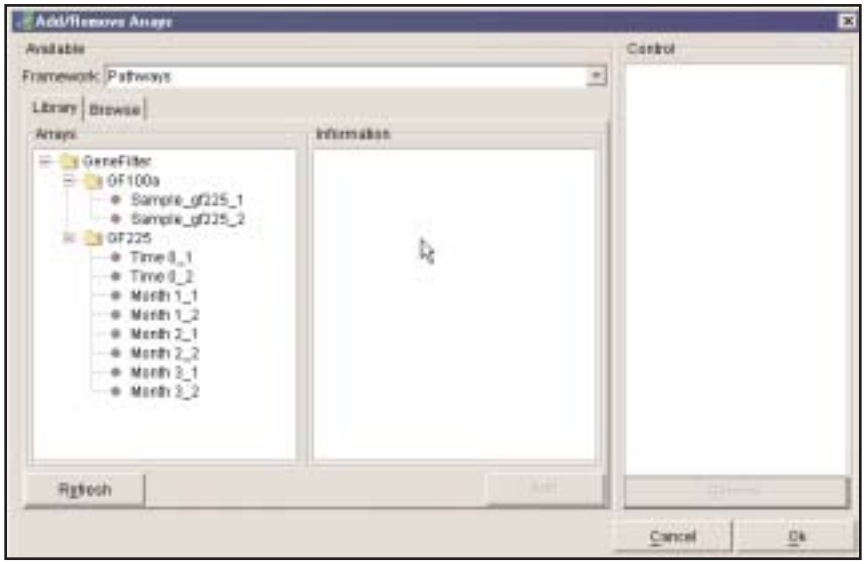
Other features may depend on the type of framework. The detail viewer may not display thumbnails with certain frameworks. Refer to Chapter 2 for more information about thumbnails. Additionally, the synthetic microarray view is unavailable if the framework does not provide geometry. Refer to Chapter 11 for more information about the synthetic view.

3.3 The Pathways™ Framework

The Pathways™ framework is the standard framework used to import microarray data for analysis. Before data reaches the Pathways™ framework, it must be processed by a Description plug-in. Description plug-ins provide a format for geometry, meta data, and other information that Pathways™ can use for analysis. ResGen™ GeneFilters® microarrays pass through the GF Description plug-in before the Pathways™ framework utilizes them. Other data types pass through the Universal Description plug-in, after the Array Designer processes them (refer to Chapter 4 for more information on the Array Designer).

Pathways™ stores a list of previously imported microarrays in a library file, organized by microarray brands and types. This library is similar to a collection of recently accessed files. The user needs only to import the data for a microarray once. After that, the data is available in the library or by browsing directly for the imported file. Use this library to set up new projects in Pathways™.

A project consists of one or more conditions, which in turn consist of one or more microarrays. The process of creating a project involves creating a condition and then adding microarrays to it. To add a microarray to the currently selected condition, choose the **Add Microarray** menu item from the **Edit** menu, or right click on the condition name. The **Add/Remove Arrays** dialog box appears.



The **Framework** field allows the user to choose a framework in which to work.

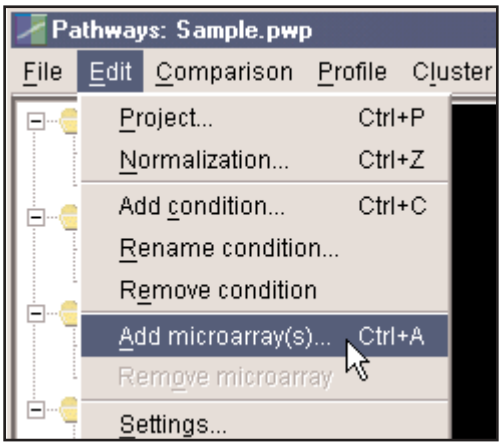
The contents of the rest of this dialog box depend on the current framework. The Pathways™ framework allows the user to add microarrays to a condition by selecting them from the library of previously imported microarrays or by browsing the file system for additional microarray files that are not in the library. Chapter 15 contains a detailed example of adding a microarray using the Pathways™ framework.

For frameworks that do not provide an import tool or a library of previously imported files, the only way to add new microarrays to the project is to browse the file system. In this case, the user's responsibilities are reduced to selecting the file and answering a few questions about how to map the data in that file to a microarray data model.

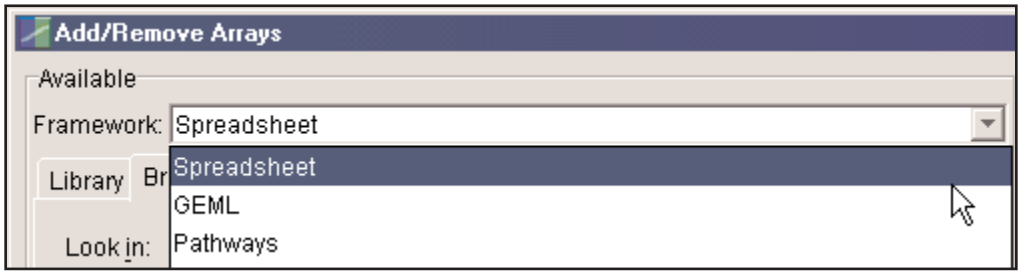
3.4 The Spreadsheet Framework

One way of adding a new microarray to a project without using an image import tool is through using the Spreadsheet Framework. The spreadsheet framework derives intensities and meta data and may optionally derive geometry from a delimited text file. In this example, an Affymetrix GeneChip® file is imported, though the process is the same for any spreadsheet.

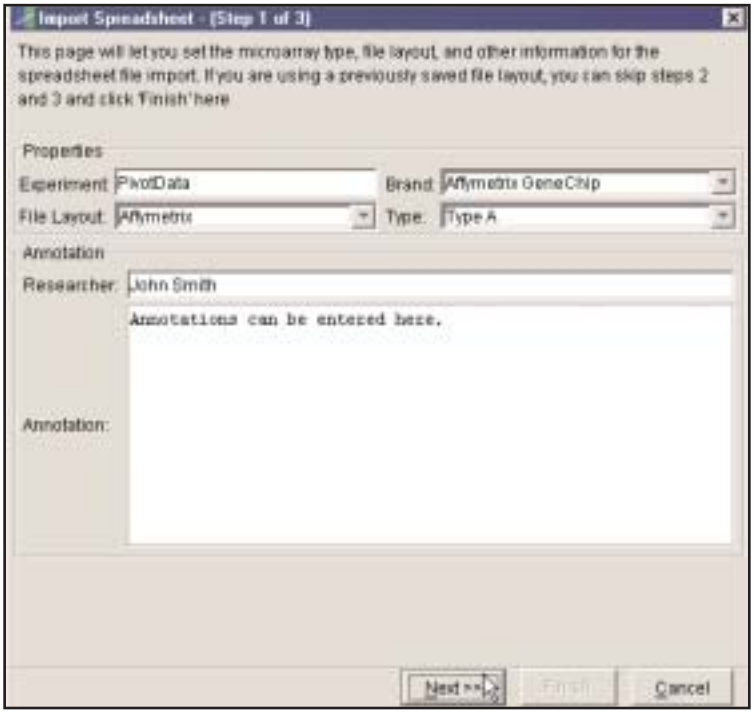
To add a microarray from a spreadsheet file, select the project condition to add the GeneChip® experiment to and select **Add microarray(s)** from the **Edit** menu.



The **Add/Remove Arrays** window appears. Select *Spreadsheet* from the Framework field at the top of the window.



Switch to the *Browse* tab to locate the GeneChip® file. Click on the file, and then click on the *Add* button. The **Import Spreadsheet** wizard appears.

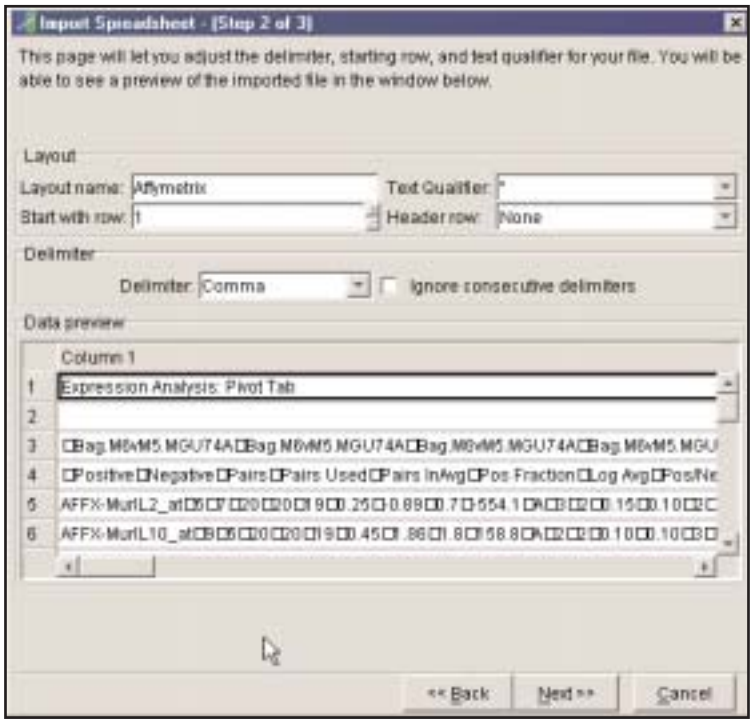

Import Spreadsheet - (Step 1 of 3)
This page will let you set the microarray type, file layout, and other information for the spreadsheet file import. If you are using a previously saved file layout, you can skip steps 2 and 3 and click 'Finish' here
Properties
Experiment: PivotData
Brand: Affymetrix GeneChip
File Layout: Affymetrix
Type: Type A
Annotation
Researcher: John Smith
Annotations can be entered here.
Annotation:
Next >>
Finish
Cancel

The first time a spreadsheet layout is being used, a profile of the layout must be created. The program remembers this profile so that future microarrays using the same spreadsheet layout may be imported without resetting the parameters. The first screen of the Import Spreadsheet wizard contains fields where the microarray **Brand**, **File Layout**, and **Type** can be set. The File Layout is the most general descriptor; it contains information about the layout of the spreadsheet. The same File Layout may be used for many different Brands. Similarly, the same Brand may contain many different Types.

In addition, **Experiment** and **Researcher** names and **Annotations** may also be entered on this screen. Once the layout has been created, click the *Next* button to continue.

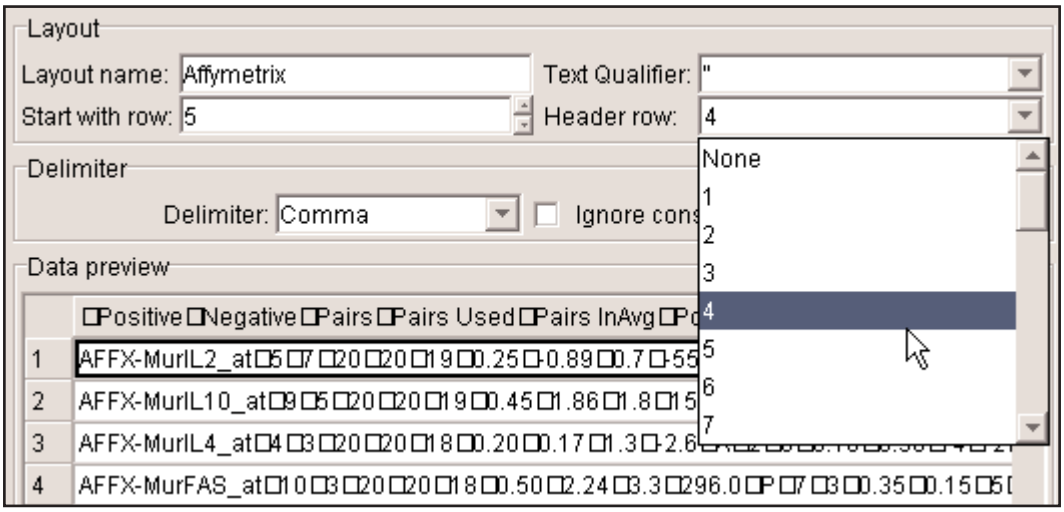
If the file layout has been created, the next two steps may be skipped by clicking the *Finish* button. The microarray is imported without changing the layout. Clicking the *Next* button will generate a window asking if the user wants to **Edit** or **Copy** the layout.

Set the layout of the spreadsheet on the second screen of the Import Spreadsheet wizard.

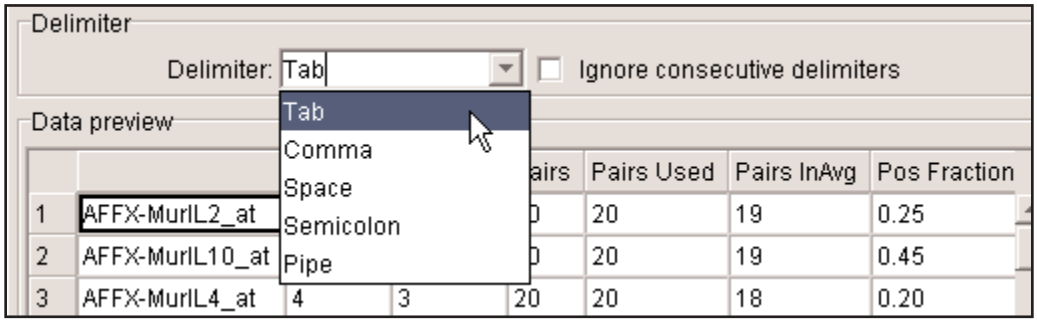


The **Layout name** field contains the name that was entered in the File Layout field on the previous screen. In the **Text Qualifier** field, select the symbol used to delineate text from the rest of the spreadsheet. Typically, this symbol is either an apostrophe (') or quotation marks ("). Select *None* if the spreadsheet does not delineate text. For Affymetrix spreadsheets, quotation marks are used as the text qualifier.

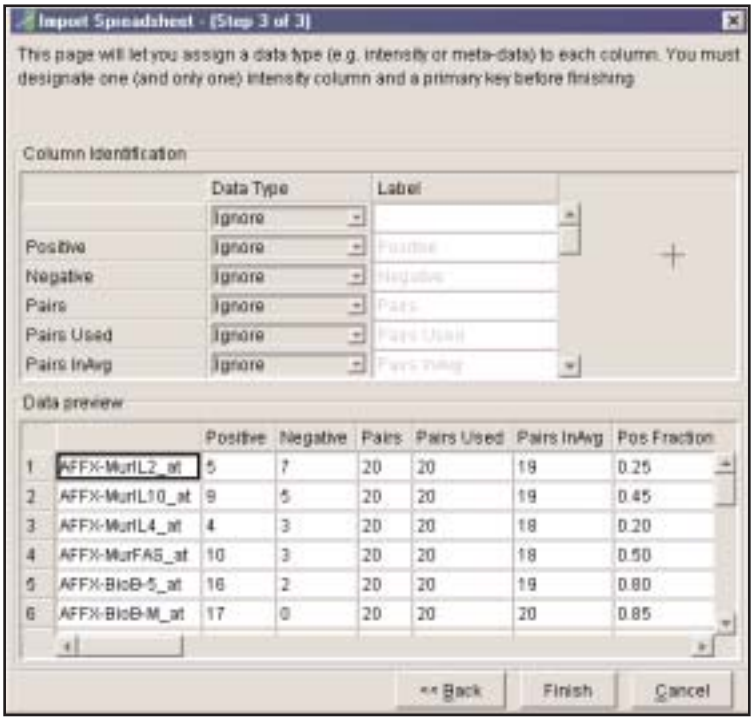
In the **Start with row** field, select the row on which the data begins. In the **Header row** field, select the row on which column headers for the spreadsheet are located, or select *None* when there is no header. Typically, the header row will be one row above the first row of data. For this example, the data start on row 5, and the header row is row 4.



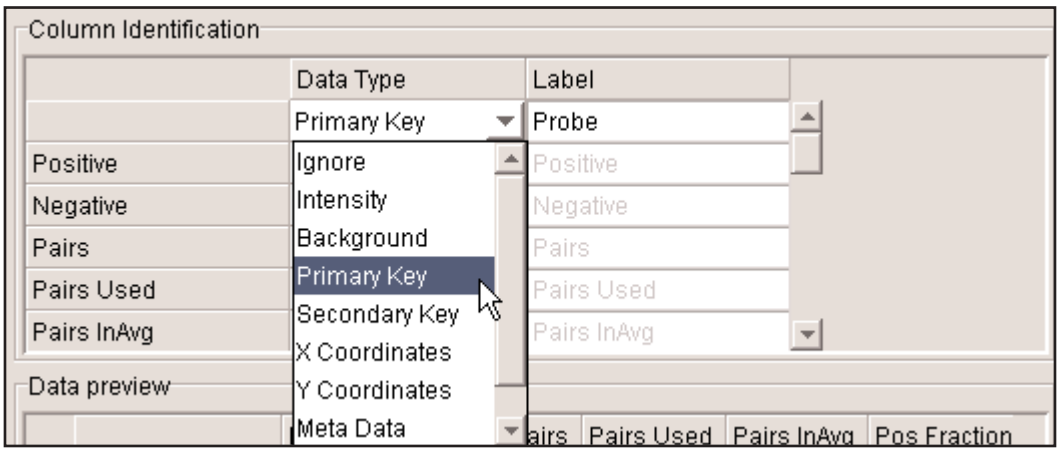
In the **Delimiter** field, select the character used to separate columns in the spreadsheet. The Affymetrix spreadsheet in this example is separated by the Tab character. If the **Ignore consecutive delimiters** box is checked, Pathways™ treats two consecutive delimiter characters as one.



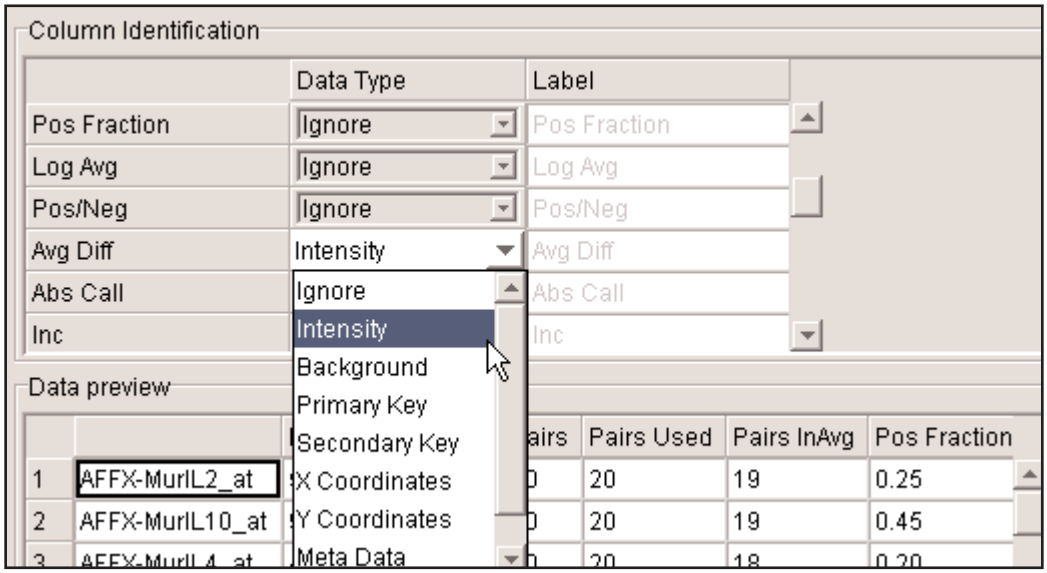
Once these fields have been selected, click the *Next* button to continue. The third screen of the Import Spreadsheet wizard appears.


Import Spreadsheet - (Step 3 of 3)
This page will let you assign a data type (e.g. intensity or meta-data) to each column. You must designate one (and only one) intensity column and a primary key before finishing.
Column Identification
Data Type
Label
Ignore
Positive
Negative
Pairs
Pairs Used
Pairs InAvg
Data preview
Positive
Negative
Pairs
Pairs Used
Pairs InAvg
Pos Fraction
1 AFFX-MurIL2_at 5 7 20 20 19 0.25
2 AFFX-MurIL10_at 9 5 20 20 19 0.45
3 AFFX-MurIL4_at 4 3 20 20 18 0.20
4 AFFX-MurFAS_at 10 3 20 20 18 0.50
5 AFFX-BioB-5_at 16 2 20 20 19 0.80
6 AFFX-BioB-M_at 17 0 20 20 20 0.85
<< Back
Finish
Cancel

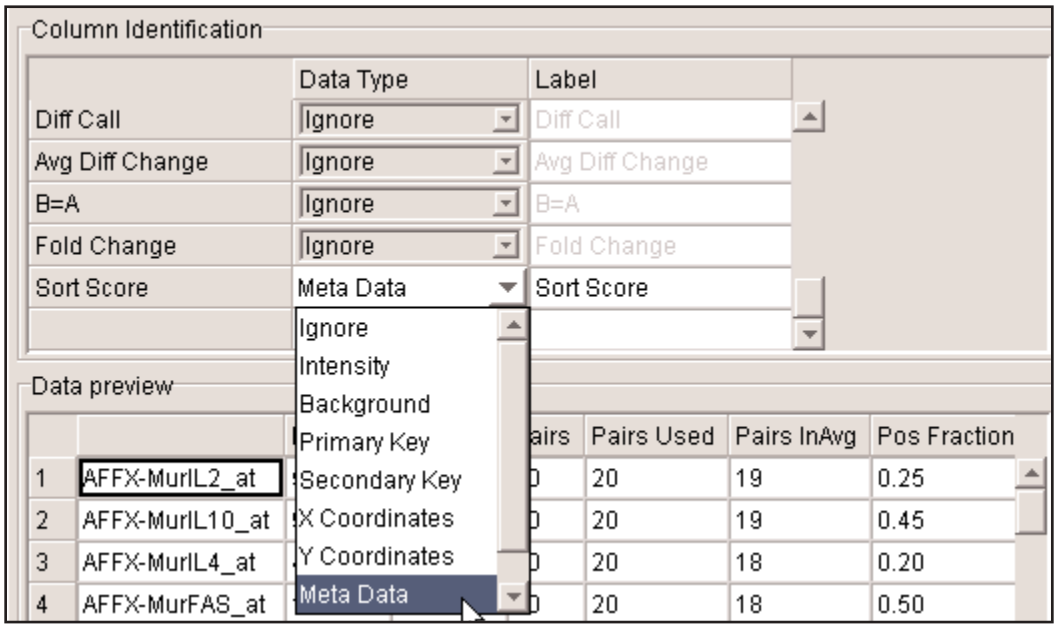
In the **Column Identification** section, a **Data Type** and a **Label** for each column may be entered. One column must be set as the **Primary Key**. The Primary Key is a unique identifier for genetic material that Pathways™ uses to distinguish between microarrays. The clone key is usually the accession number or image ID of the clone although the key could follow any other naming convention. As discussed in Chapter 9, the key field is one way of grouping similar clones together for statistical analysis. A **Secondary Key** mst be specified if the spreadsheet has any missing Primary Key entries. Refer to Chapter 7 for more information on keys. In this example, the first column is used as the Primary Key for Affymetrix spreadsheets.



One column must be set as the **Intensity**. The average difference (**Avg Diff**) column is used as the Intensity setting for this example.



The last column of the example Affymetrix spreadsheet file contains a text description of the experiment. Set the data type for this column to **Meta Data** to see it during analysis. The label for Meta Data may be edited by the user. In addition, the data type for additional columns may be set to Meta Data to view those columns during analysis.



A spreadsheet may provide geometry in the form of X and Y coordinates. If it does so, Pathways™ will create a synthetic view of the microarray during the importing process. Set the appropriate columns to specify X and Y coordinates for a layout.

The **Data Preview** section of the window contains the finished layout of the spreadsheet. If the spreadsheet is improperly laid out, click the *Back* button to change its parameters. To finish importing the spreadsheet, click the *Finish* button.

3.5 The GEML™ Framework

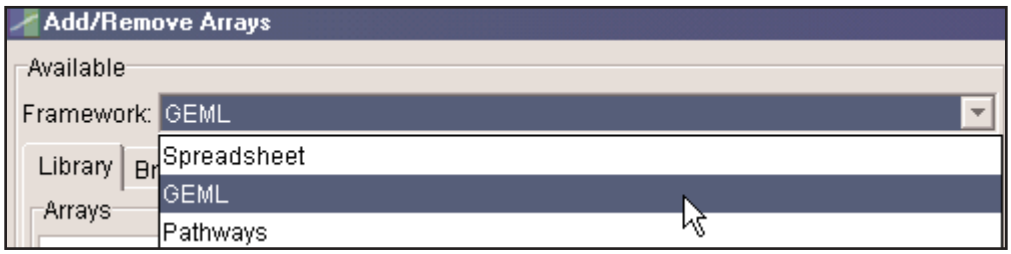
The Extensible Markup Language (XML) is a syntax format created by the World Wide Web Consortium for creating customized markup languages. XML can be used to create tags that focus on a particular type of data. XML-based tag sets are designed according to their content, allowing them to provide specific information about their content and how that content relates to other data. They are self-describing, eliminating the need to include extraneous documentation when transmitting data.

The **Gene Expression Markup Language (GEML™)** is an XML-based tag set that provides a method for exchanging gene expression data and related annotations. Rosetta Inpharmatics developed GEML™ as a means of transmitting data between different gene expression systems, databases, and tools. GEML™ separates data collection and reporting from the methodology used to collect and report that data, enabling the analysis of data derived from differing methodologies through the use of the same syntax.

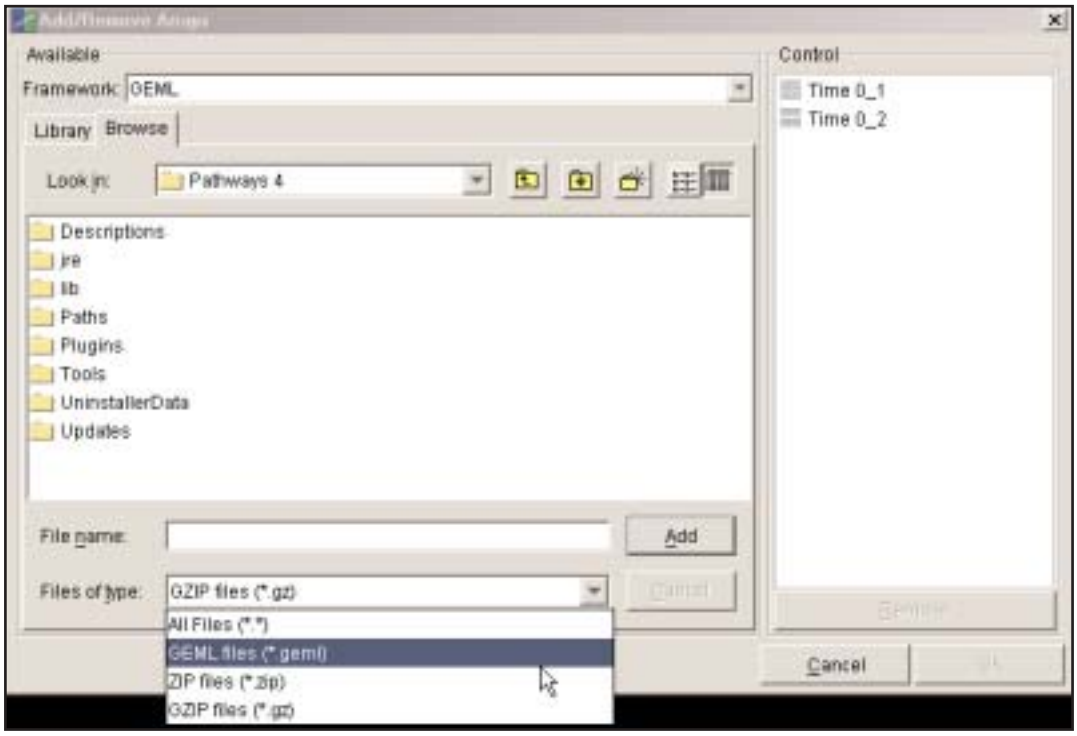
Pathways™ Universal features a GEML™-compatible framework that takes GEML™ files and translates them into a format the program can use in the analysis of data. Pathways™ processes GEML™ files directly with the GEML™ framework. Other gene expression data files can be converted to GEML™ files through a program like the Rosetta GEML Conductor™.

The GEML™ framework does not provide an import tool, so new microarrays must be added by browsing the file system. In this way, adding a GEML™ file is similar to adding a spreadsheet file.

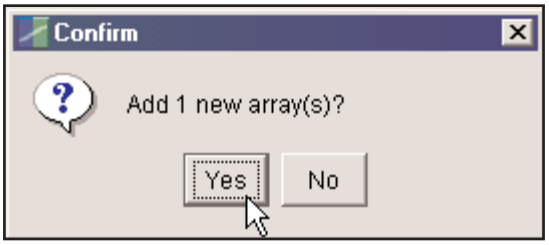
To add a new microarray to the project from a GEML™ file, select the project condition to add the experiment to and select **Add Microarray(s)** from the **Edit** menu. The **Add/Remove Arrays** window appears. Select **GEML** from the **Framework** field at the top of the window.



Switch to the *Browse* tab to locate the file in the system. The GEML™ framework can automatically decompress ZIP and GZIP files in addition to opening GEML™ files, so make sure the **Files of type** field is set appropriately.



Select the GEML™ file, click the *Add* button, and then click the *Ok* button to continue. A window will appear asking for confirmation.



To finish importing the microarray from the GEML™ file, click the *Yes* button.

Chapter 4: The Array Designer

4.1 Introduction

Previous versions of Pathways™ imported a single microarray type, ResGen™ GeneFilters®. Pathways™ Universal introduces the Array Designer feature, which facilitates the import of any microarray type as long as certain data is known about the array layout. Using the Array Designer is a simple matter of telling Pathways™ some information about the type of microarray to be used.

Different microarray types use different array layouts. To import an image, Pathways™ must know what kind of array layout to expect. **The Array Designer allows the user to give the program the layout information it needs to import a microarray image for use in analysis.** During the analysis process, the user may want to know more information about a microarray spot with a certain intensity. **The Array Designer allows the user to associate descriptive information (meta data) with intensity for each microarray spot in the layout.**

The Array Designer provides customized microarray layouts for GEML™ files, Clontech Atlas™ Array Gene List files, and Corning CMT™ Map files. It can also design layouts for any other type of microarray that is described in a spreadsheet format. **The spreadsheet must include the X and Y coordinates for the array layout and the associated meta data for each spot on the microarray.** The Array Designer takes this information from the spreadsheet file and creates a visualization of the microarray, which can then be imported for analysis.

When a layout is designed using the Array Designer, the user will enter the **Brand** and **Type** of the microarray. A microarray brand is usually the name of the product line (*e.g.* GeneFilters®), while a microarray type is the specific kind of microarray from that product line (*e.g.* GF200). A specific brand may contain many different types. Once the Array Designer creates a layout for a particular type of microarray, Pathways™ stores information about that microarray in description files. These description files are located in the installation directory in the folder **Descriptions\Universal\<Brand>**, where <Brand> is the user-inputted name of the product line.

The user needs to design a layout for a particular microarray type only once. After that, the user can import images of this microarray type multiple times without having to design a new layout.

4.2 Concepts: Importing

Microarray intensity levels must be measured before a microarray experiment can be analyzed. The importing process begins with a raw image (Tiff, Gel, Fuji, etc.) of a microarray and ends with sampled intensity levels for each spot present on the microarray.

The Array Designer allows the user to provide Pathways™ with the information necessary to import and sample microarray images. This information includes the physical location of the spots on a reference (ideal) layout and descriptive information (meta data) for each spot. Once the array design has been provided to Pathways™, a description of the design will be stored on the computer's hard drive, and this description will be used in importing and analysis of the given brand and type.

Before the spot intensity can be sampled, it is necessary to determine the location of each spot on the experimental image. There are two primary steps in the spot centering process:

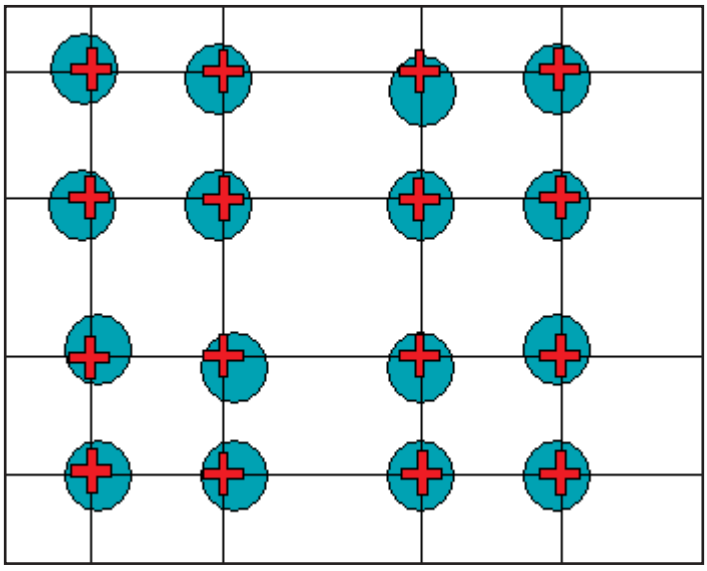
1. **Determining seed locations for the spots**

2. **Refining the seed locations to more exactly match the experimental image**

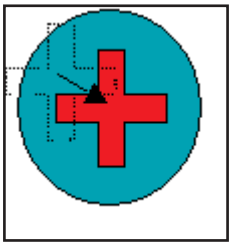
Each of these steps is discussed in more detail below.

4.3 Concepts: Auto/Crop Mode

The Pathways™ importer has a built-in capability for determining the skew (angulation) of microarray images and then searching for the rows and columns that are typically present in microarray layouts. These rows and columns can be used to automatically generate seed locations for each spot, based upon the individual spot's row and column in the reference geometry provided to the Array Designer. This concept is illustrated below (the crosses represent the seed locations which would be determined for these rows and columns).



Seed locations will not necessarily find the exact location of the spot centers. Therefore, each seed location is independently adjusted using a user-specified **autocenter** mode.



The three autocenter modes which are available in the current release of Pathways™ are Centroidal, Profile, and None. These modes will be discussed in more detail later in this chapter.

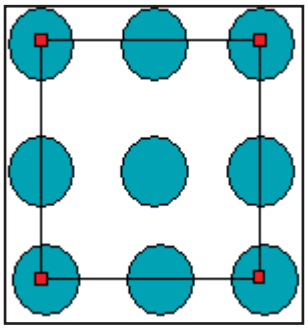
The Pathways™ importer allows a **cropping window** to be specified. The purpose of this cropping window is to reduce the region of the image which will be searched during the auto alignment process. This cropping window should not be confused with the template **bounding box**, which is described later in this chapter.

The Pathways™ importer only requires the x and y location of the spot centers in a reference layout and the choice of autocenter mode in order to execute the auto/crop mode for determining centers.

4.4 Concepts: Template Mode

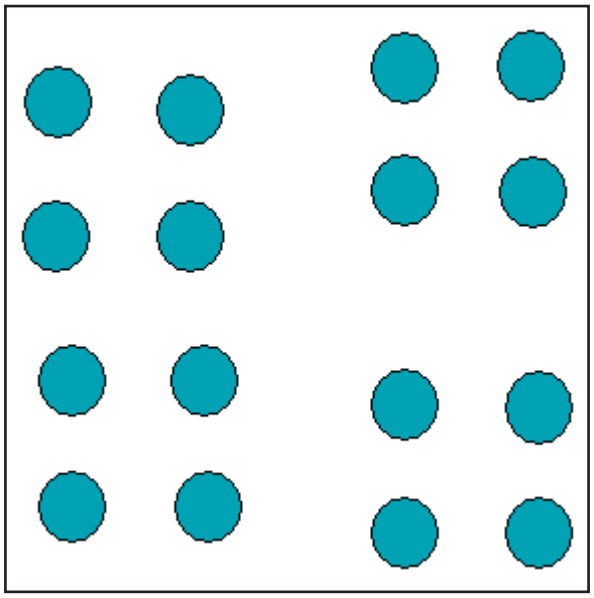
Sometimes noise levels in the image or other characteristics of an experimental image prevent the auto/crop mode from successfully aligning the image. If this is the case, the user will manually generate the seed locations for the spots using **templates**. Templates are sketches of the ideal position of the spots which are superimposed upon the experimental image in the Pathways™ importer to generate the initial seed positions. All templates are tied to a global bounding box which is used for initial sizing and positioning of the templates. This global bounding box is by default anchored at the minimum and maximum positions on the microarray, but can be set by the user to, for example, lie on top of control points on the user's array. Once the global adjustments are complete, each point on each template can be individually fine tuned before generating the seed locations.

In a simple microarray geometry, such as the one shown below, the user may choose to have a single template that lies on top of the global bounding box (this is the default configuration for the Array Designer).

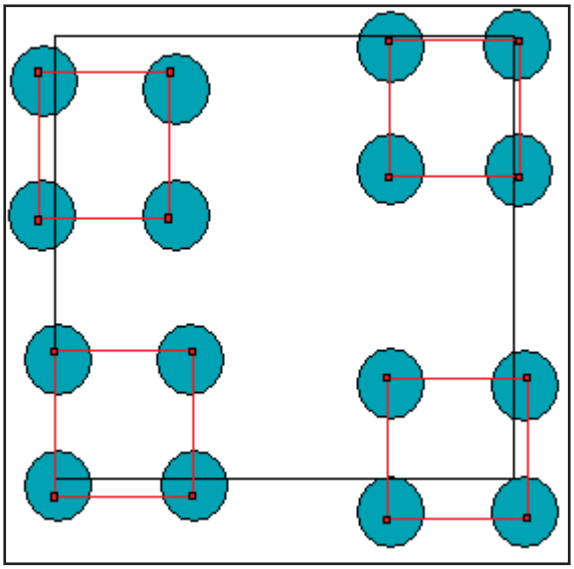


Once this template is overlaid on the experimental image, Pathways™ will generate the seed positions for each spot based upon a comparison of the template in the ideal coordinates versus the position of the template in the overlay in the experimental image.

Often the manufacturing process for microarrays introduces known offsets in spot positions based upon the mechanical devices that create the microarray. For example, each subgrid in a microarray layout with multiple subgrids might be offset from the other subgrids due to the manufacturing process.

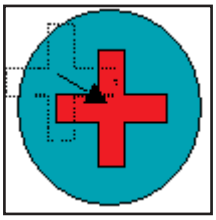


If this is the case, then it is advisable that each subgrid be assigned its own template in the Array Designer (in the figure below, templates are shown in red, global bounding box in black).



In general, the smallest number of templates needed to adequately describe the geometry should be used because each template may require further individual adjustment by the user during the import process.

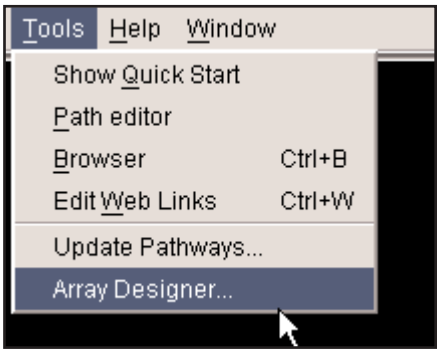
Regardless of the number of templates, the final portion of the template importing process is identical to the auto/crop mode. The seed positions which are generated from the template overlay are fine tuned according to the autocenter mode choice.



For more information on the autocentering and the importing process, refer to Chapters 5 and 6.

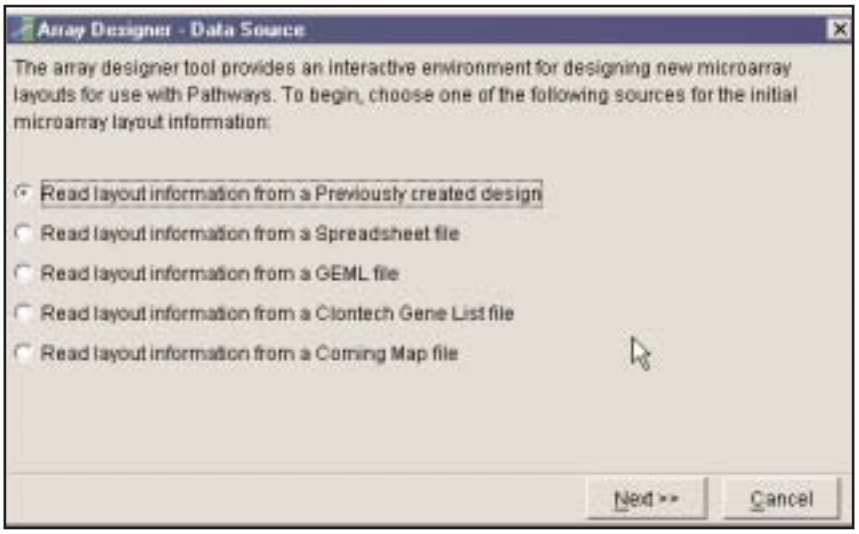
4.5 Opening the Array Designer

To open the Array Designer, select **Array Designer** from the **Tools** menu, or click the bottom button in the Quick Start menu.



Pathways Quick Start
Import a new microarray image
Open an existing project
Open the project wizard to guide you through the creation of a new project
Analyze relative intensity values of a single microarray
Compare two microarrays using intensity ratios and differences
Construct a dynamic list of genes built on your criteria
Design new microarray layouts using the Array Designer tool
Array Designer
Close

The **Array Designer - Data Source** window appears.


Array Designer - Data Source
The array designer tool provides an interactive environment for designing new microarray layouts for use with Pathways. To begin, choose one of the following sources for the initial microarray layout information:
Read layout information from a Previously created design
Read layout information from a Spreadsheet file
Read layout information from a GEML file
Read layout information from a Clontech Gene List file
Read layout information from a Corning Map file
Next >>
Cancel

WARNING

Pathways™ must be restarted if a microarray description which has been loaded into the current Pathways™ session (*e.g.* the description has been used within a project or within the importer) is modified. Changes made in the Array Designer will not take effect until Pathways™ is restarted.

If a layout has already been defined for the type of microarray layout being designed, select **Read layout information from a Previously created design**, and click the *Next* button. A window appears, and it asks for the location of the description file.

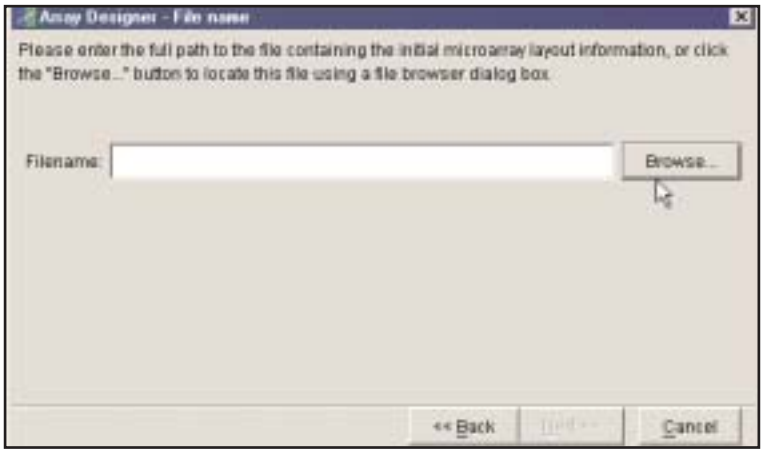
Array Designer - Description file name
Please enter the full path to the file containing a Pathways Universal description file, or click the "Browse..." button to locate this file using a file browser dialog box.
Filename:
Browse...
<< Back
Cancel

Click the *Browse* button to locate the description file in the system. Pathways™ creates a description of the attributes of every microarray layout designed with the Array Designer. The program can then access these files quickly for future use. Description files must be stored in the **Descriptions\Universal\<Brand>** folder in the Pathways™ installation directory, where <Brand> is the entered name of the product line.

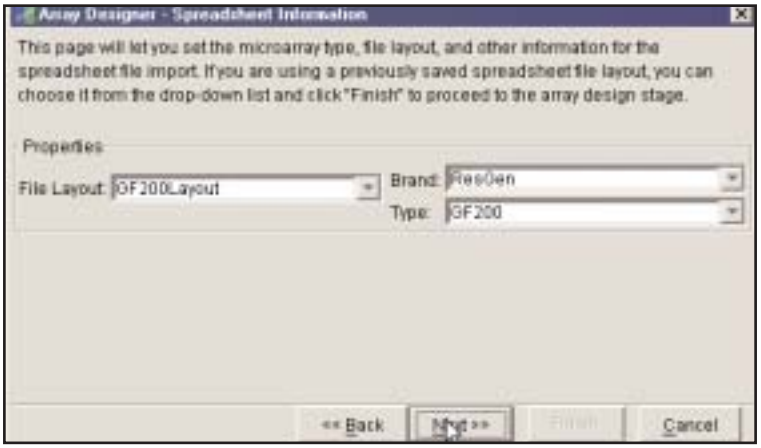
4.6 Reading from a Spreadsheet File

The Array Designer provides customized microarray layouts for GEML™ files, Clontech Atlas™ Array Gene List files, and Corning CMT™ Yeast Array Map files. When the microarray data is in none of these formats, a layout for the microarray must be designed from a spreadsheet file.

To design a layout for a spreadsheet, select **Read layout information from a Spreadsheet file** from the **Array Designer - Data Source** window, and click the *Next* button. A window appears, and it asks for the file location.


Array Designer - File name
Please enter the full path to the file containing the initial microarray layout information, or click the "Browse..." button to locate this file using a file browser dialog box.
Filename:
Browse...
<< Back
Cancel

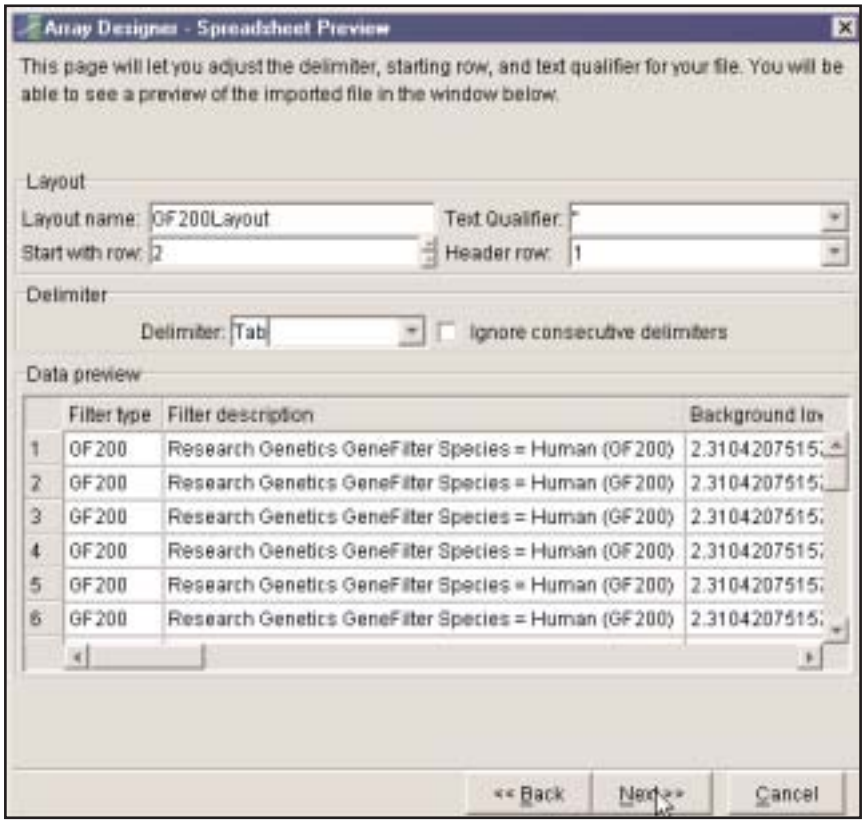
Click the *Browse* button to locate the file in the system. Once the file has been located, click the *Next* button. A window appears, and it asks for information about the spreadsheet.


Array Designer - Spreadsheet Information
This page will let you set the microarray type, file layout, and other information for the spreadsheet file import. If you are using a previously saved spreadsheet file layout, you can choose it from the drop-down list and click "Finish" to proceed to the array design stage.
Properties
File Layout: GF200Layout
Brand: ResGen
Type: GF200
<< Back
Next >>
Finish
Cancel

In this window, enter the microarray **File Layout**, **Brand**, and **Type**. The name entered in the **File Layout** field is used to signify a set of attributes for the spreadsheet. The name entered in the **Brand** field is the name of the product line, and the name entered in the **Type** field is the kind of microarray from the product line.

Pathways™ stores this information and creates a profile for the spreadsheet layout, allowing the researcher to quickly import a microarray that uses the same layout. If a spreadsheet has been defined for the microarray being imported, click the *Finish* button to proceed to the array design stage.

If a microarray layout is being designed for the first time, click the *Next* button to define the attributes of the spreadsheet in the **Spreadsheet Import** wizard.

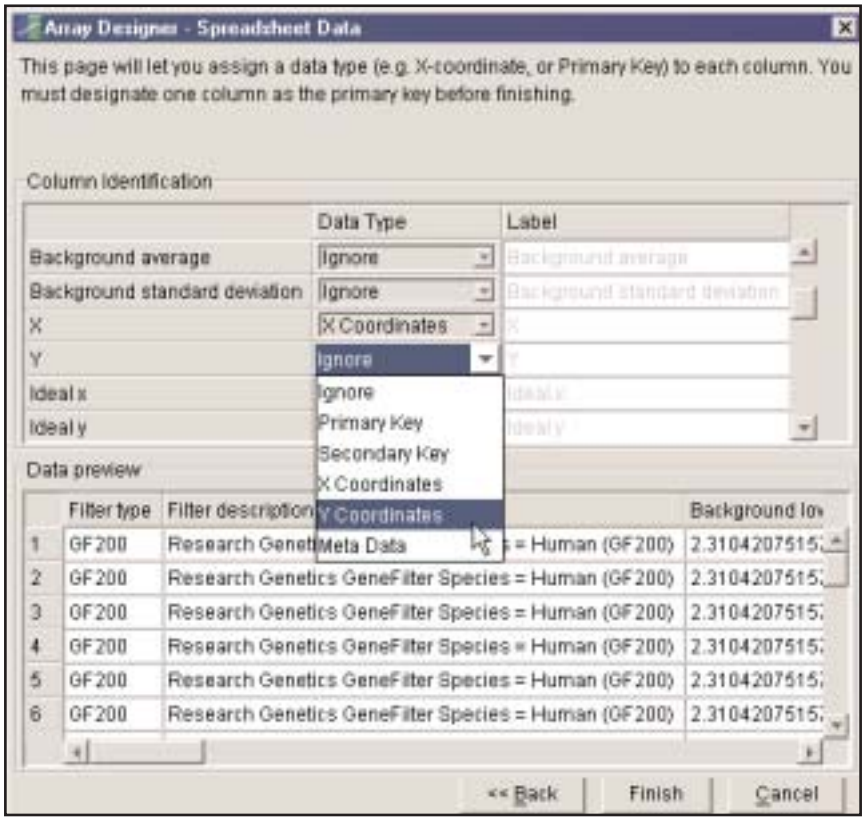

Array Designer - Spreadsheet Preview
This page will let you adjust the delimiter, starting row, and text qualifier for your file. You will be able to see a preview of the imported file in the window below.
Layout
Layout name: GF200Layout
Text Qualifier: "
Start with row: 2
Header row: 1
Delimiter
Delimiter: Tab
Ignore consecutive delimiters
Data preview
Filter type
Filter description
Background Io
1 GF200 Research Genetics GeneFilter Species = Human (GF200)
2 GF200 Research Genetics GeneFilter Species = Human (GF200)
3 GF200 Research Genetics GeneFilter Species = Human (GF200)
4 GF200 Research Genetics GeneFilter Species = Human (GF200)
5 GF200 Research Genetics GeneFilter Species = Human (GF200)
6 GF200 Research Genetics GeneFilter Species = Human (GF200)
<< Back
Next >>
Cancel

The **Layout name** field contains the name that was entered in the File Layout field on the previous screen. In the **Text Qualifier** field, select the symbol used to delineate text from the rest of the spreadsheet. Typically, this is either an apostrophe (') or quotation marks ("). Select *None* if the spreadsheet does not delineate text.

In the **Start with row** field, enter the row on which the data begins. In the **Header row** field, enter the row on which column headers for the spreadsheet are located, or enter *None* when there is no header. Typically, the header row is one row above the first row of data.

In the **Delimiter** field, select the character used to separate columns in the spreadsheet. If the **Ignore consecutive delimiters** box is checked, Pathways™ treats two consecutive delimiter characters as one.

Once these fields have been selected, click the *Next* button. The next window of the Import Spreadsheet wizard appears.


Array Designer - Spreadsheet Data
This page will let you assign a data type (e.g. X-coordinate, or Primary Key) to each column. You must designate one column as the primary key before finishing.
Column Identification
Data Type
Label
Background average
Ignore
Background standard deviation
Ignore
X
X Coordinates
Y
Ignore
Ideal x
Ideal y
Ignore
Primary Key
Secondary Key
X Coordinates
Y Coordinates
Meta Data
Data preview
Filter type
Filter description
Background lo
1 GF200 Research Genet = Human (GF200) 2.3104207515
2 GF200 Research Genetics GeneFilter Species = Human (GF200) 2.3104207515
3 GF200 Research Genetics GeneFilter Species = Human (GF200) 2.3104207515
4 GF200 Research Genetics GeneFilter Species = Human (GF200) 2.3104207515
5 GF200 Research Genetics GeneFilter Species = Human (GF200) 2.3104207515
6 GF200 Research Genetics GeneFilter Species = Human (GF200) 2.3104207515
<< Back
Finish
Cancel

In the **Column Identification** section, a **Data Type** and a **Label** for each column may be entered.

When designing a layout for a microarray image with the Array Designer, the user must indicate which columns are to be used for X and Y Coordinates. When these columns are not specified, the program lacks the geometry data that it needs to construct an image for importing.

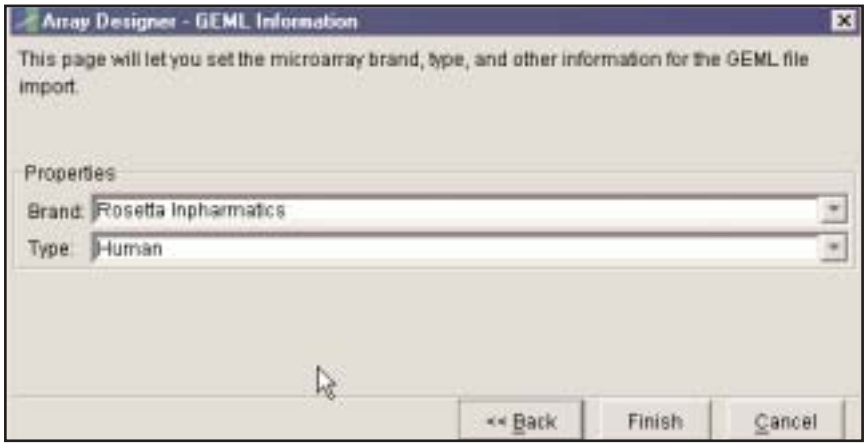
One column must be set as the **Primary Key**. The Primary Key is a unique identifier for genetic material that Pathways™ uses to distinguish between microarrays. The clone key is usually the accession number or image ID of the clone although the key could follow any other naming convention. As discussed in Chapter 9, the key field is one way of grouping similar clones together for statistical analysis. A **Secondary Key** mst be specified if the spreadsheet has any missing Primary Key entries. Refer to Chapter 7 for more information on keys. The user may edit the **Label** field for Primary and Secondary Keys.

Any column containing descriptive information can be set as **Meta Data**. When a column's Data Type is set as Meta Data, the column information for each spot is available during the analysis process. The user may edit the **Label** field for Meta Data.

The **Data Preview** section of the window contains the finished layout of the spreadsheet. If the spreadsheet is improperly laid out, click the *Back* button to change its parameters. To proceed to the Array Design stage, click the *Finish* button.

4.7 Reading from a GEML™ File

To design a microarray layout for a GEML™ file, open the Array Designer and select **Read layout information from a GEML file** from the **Array Designer - Data Source** window. Then click the *Next* button. A window appears asking for the file location. Click the *Browse* button to locate the file in the system. Once the file has been located, click the *Next* button. A window appears, and it asks for information about the GEML™ file.

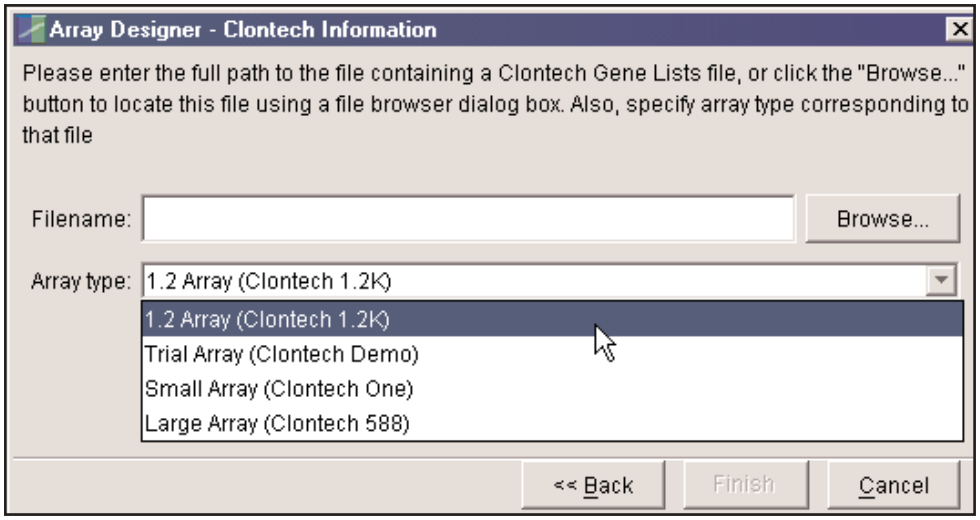

Array Designer - GEML Information
This page will let you set the microarray brand, type, and other information for the GEML file import.
Properties
Brand: Rosetta Inpharmatics
Type: Human
<< Back
Finish
Cancel

In this window, enter the microarray **Brand** and **Type**. Pathways™ stores this information and creates a profile for GEML™ files of this type. Once the microarray Brand and Type have been entered, click the *Finish* button to proceed to the array design stage.

4.8 Reading from a Clontech Atlas™ Array Gene List File

To design a microarray layout for a Clontech Gene List file, open the Array Designer, and select **Read layout information from a Clontech Gene List file** from the **Array Designer - Data Source** window. Then click the *Next* button.

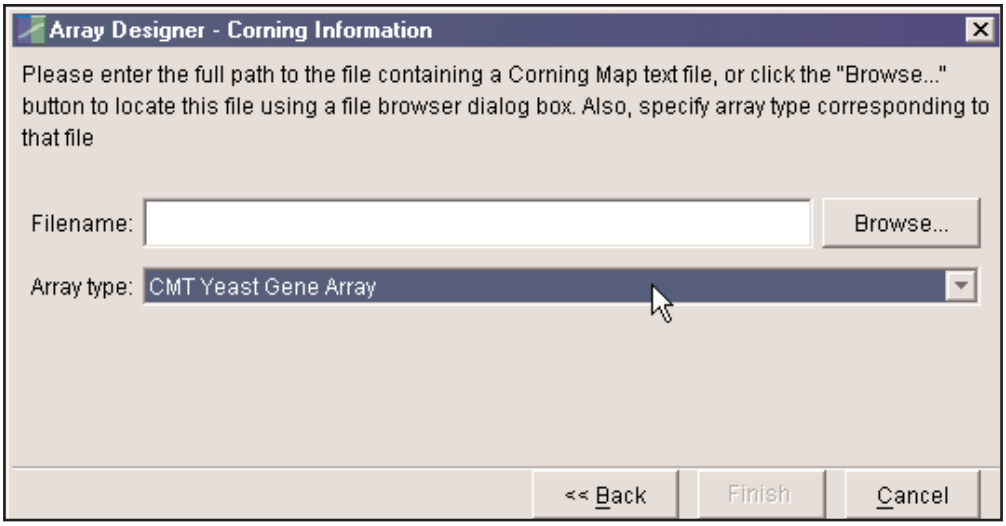
A window appears, and it asks for the file location and for the **Array Type** of the gene list file.


Array Designer - Clontech Information
Please enter the full path to the file containing a Clontech Gene Lists file, or click the "Browse..." button to locate this file using a file browser dialog box. Also, specify array type corresponding to that file
Filename:
Browse...
Array type:
1.2 Array (Clontech 1.2K)
1.2 Array (Clontech 1.2K)
Trial Array (Clontech Demo)
Small Array (Clontech One)
Large Array (Clontech 588)
<< Back
Finish
Cancel

Select the appropriate Array Type for the gene list file. The Array Designer currently supports Clontech **1.2 Arrays**, **Trial Arrays**, **Small Arrays**, and **Large Arrays** (see http://www.clontech.com/atlas/index.shtml for more information about these array types). The Brand is automatically set as Clontech, and the Type is derived from the filename. Click the *Browse* button to locate the file. Once the file is located, click the *Finish* button to proceed to the array design stage.

4.9 Reading from a Corning CMT™ Map File

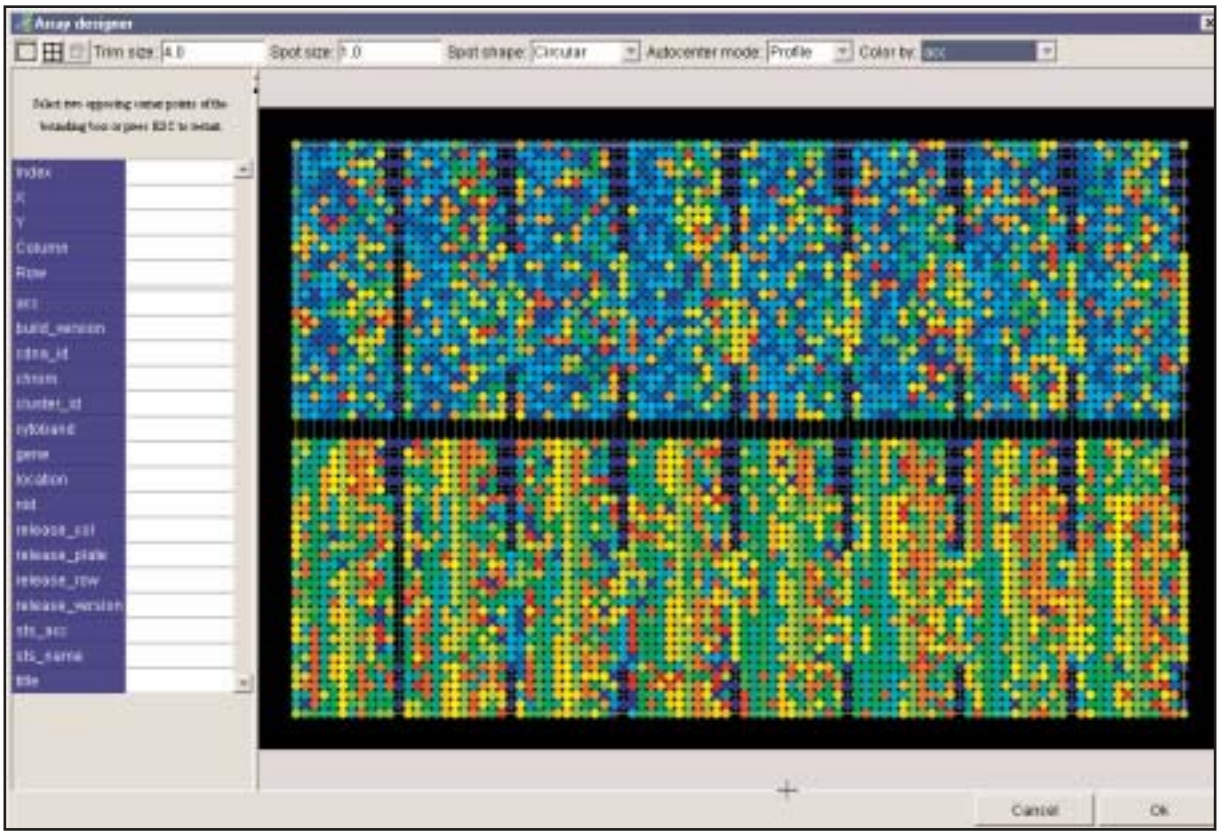
To design a microarray layout for a Corning CMT™ Map file, open the Array Designer and select **Read layout information from a Corning Map file** from the **Array Designer - Data Source** window. Then click the *Next* button. A window appears, and it asks for the file location and for the **Array Type** of the file.


Array Designer - Corning Information
Please enter the full path to the file containing a Corning Map text file, or click the "Browse..." button to locate this file using a file browser dialog box. Also, specify array type corresponding to that file
Filename:
Browse...
Array type:
CMT Yeast Gene Array
<< Back
Finish
Cancel

At the time of printing, Corning supports only **CMT™ Yeast Gene Arrays**. Future plug-ins will provide support for other Corning formats as they become available. Click the *Browse* button to locate the file. Once the file is located, click the *Finish* button to proceed to the array design stage.

4.10 The Array Design Stage

After the layout information is specified, the **Array Designer** window appears.



This window creates a view of the microarray from the layout information in the file. The fields along the top of the window allows the user to adjust certain attributes of the microarray and of the importing process.

Trim size allows the user to set the amount of space to trim from the edge of the image. If the user specifies a Trim size, trimming occurs after centering during the import process. The designated Trim size is cropped from the edge of the imported image. The units used to calculate this space are the same as the units in the input file (*e.g.* pixels). **Spot size** allows the user to set the size of the microarray spots, using the same units as the input file. **Spot shape** allows the user to set whether the microarray spots should be circular or rectangular. Both Spot size and Spot shape are considered during the import process, after centering has been completed.

The **Autocenter mode** allows the user to set the mode to use to find the centers of spots. When a microarray is imported, the program calculates a seed position for the center of each spot. However, many factors can introduce noise into the image, causing the positioning of centers to be slightly off. When the microarray image is clean and uniform, the Autocenter mode can be set to *None* to keep the positions of the centers where they are in a rigid grid pattern. There are two other centering modes that can be used to recalculate the positions of spot centers.

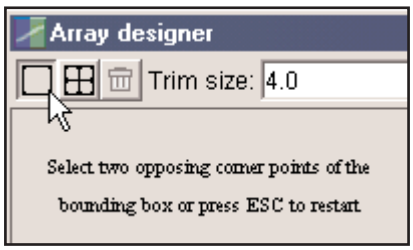
- **Profile: Profile autocentering scans the area around each spot and finds a peak intensity both horizontally and vertically. The two intensities are cross-matched, finding the area of highest intensity, which is usually the center of the spot. This mode is useful when the image is high contrast.**

- **Centroid: Centroid autocentering scans the area immediately around the current position of the spot center. The center position is moved towards the nearest area of higher intensity. This movement occurs until all the areas around the center position are of lower intensity. This mode is useful when the image is low contrast.**

The user may need to experiment with different Autocenter modes to find what works best.

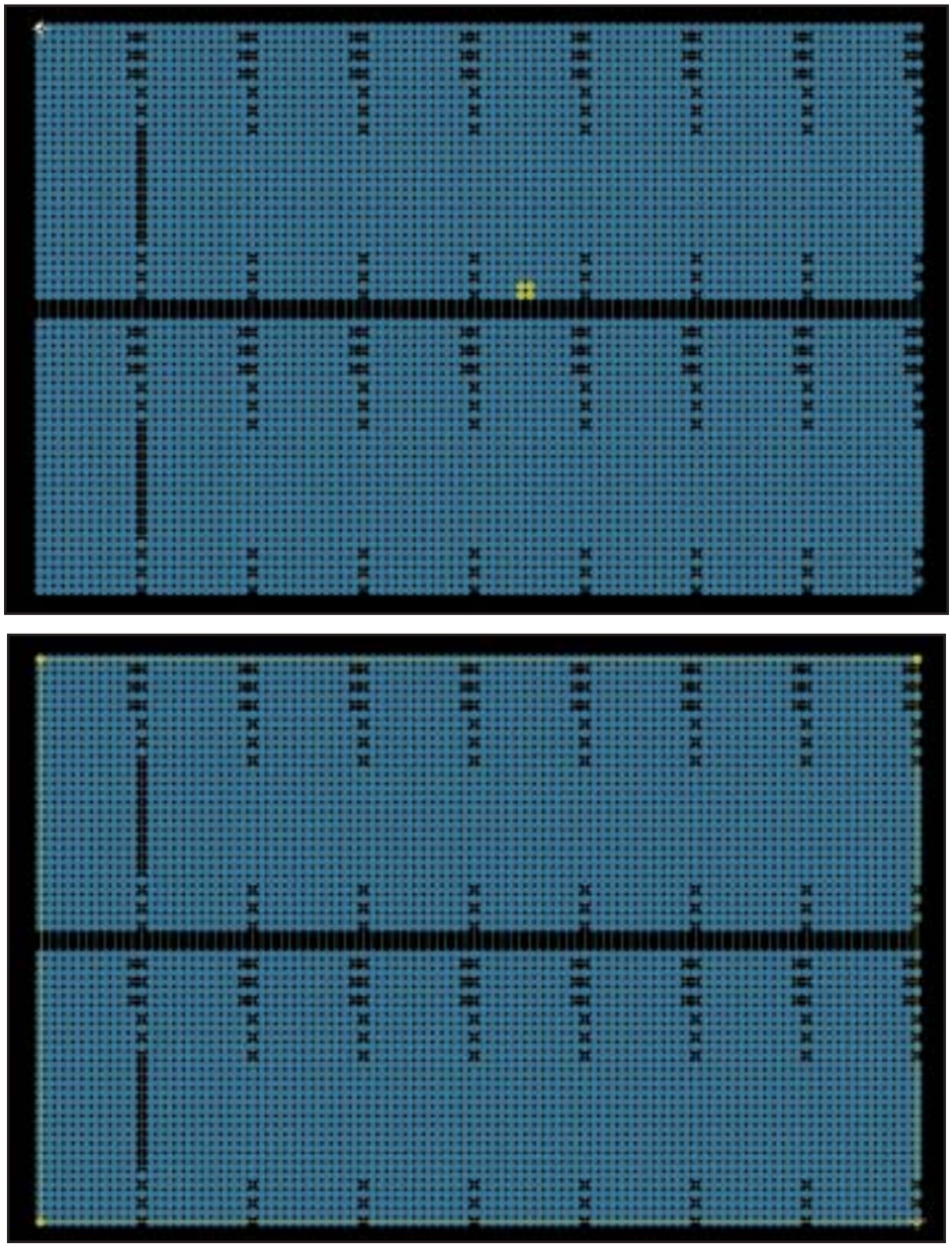
The **Color by** field allows the user to set which microarray attribute to use in differentiating color between the spots in the image, allowing for more accurate visual comparisons when creating a bounding box or template. In addition, the user may set the field to *None* to show the location of the spots. The Color by field affects only the image during the layout design stage.

In the upper left section of the window, three buttons appear. The first button allows the user to create a **bounding box**.



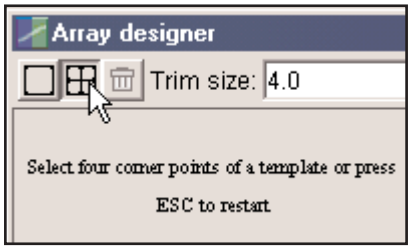
A bounding box is automatically created around the edges of the image when the layout is designed. The bounding box serves as a global template for an array layout. It tells the program where to look for other templates. When it is not properly aligned, the user may create a new one manually.

To create a new bounding box, click on two places on opposite corners of the area to be bounded.

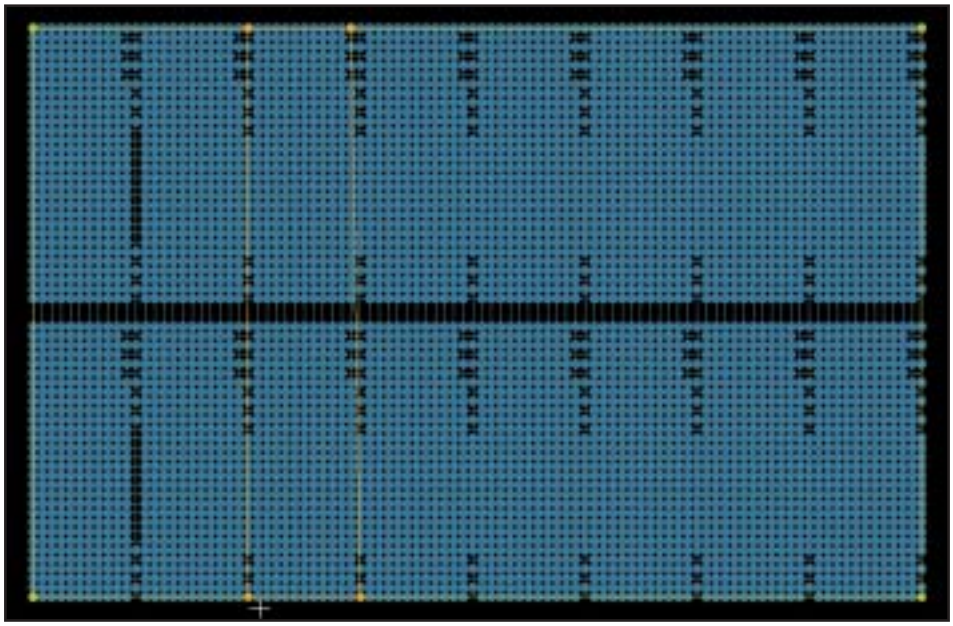


The bounding box appears. The bounding box provides Pathways™ with a rough sizing estimate for any templates that may be present.

The second button in the upper left section of the Array Designer window allows the user to create a **template**.

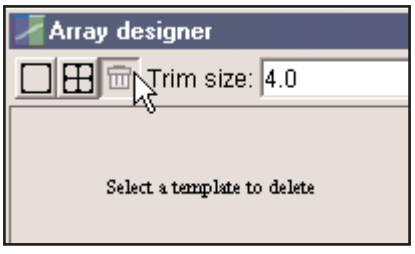


Sometimes, microarrays are laid out in sections. Templates allow the user to mark the boundaries of these sections so that the program recognizes that the microarray is not uniformly arranged (for more information on templates, refer to Chapter 6 and to Sections 4.3 and 4.4). To create a template around a section of a microarray, click the four corners of the section.

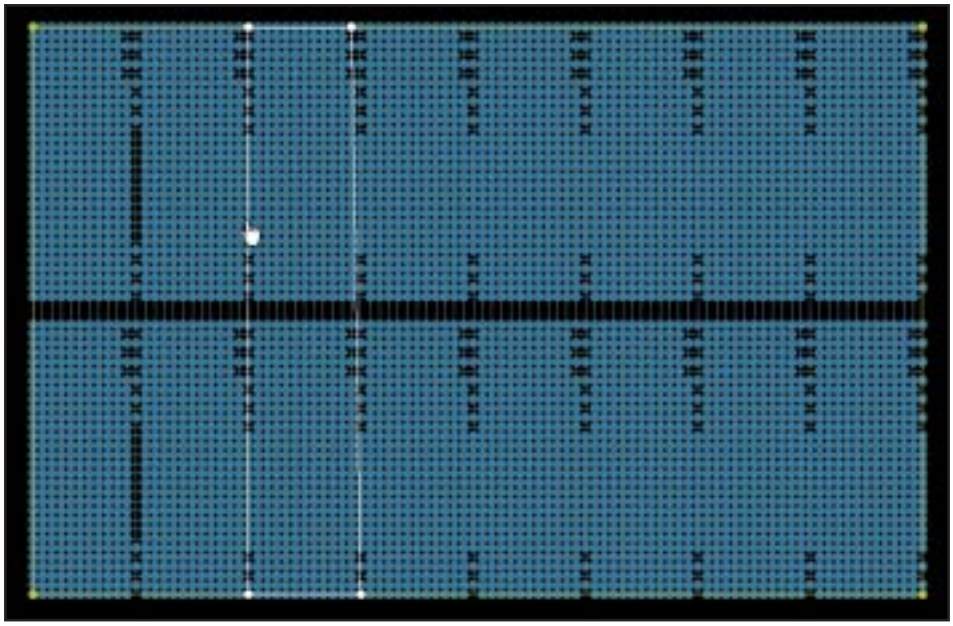


The template appears around the section.

The third button from the left in the Array Designer window allows the user to delete a template.



To delete a template, first move the cursor over one of the edges of the template.



The selected template is highlighted. Click on the template to delete it.

Once the bounding box is set and necessary templates have been created, click the *Ok* button to finish designing the microarray layout. The microarray is ready to be imported into Pathways™.

Book III: Core Concepts

Data Flow and Importing Images

Chapter 5: Data Flow - From Experiment to Analysis

This chapter describes how the Pathways Framework imports and stores data. Other Frameworks may use alternate methods to import and store data.

5.1 From Experiment to Analysis: The Importing Process

Microarray intensity levels must be measured before analysis of the microarray experiment can begin. The Pathways™ 4 **image importing process** begins with a microarray image and ends with sampled intensity levels for the clones. There are five steps in the process of importing an image file.

1 **Loading the image**
Pathways reads the image file and displays it on the screen.

2 **Aligning / cropping / template**
Adjust the image orientation and optionally apply a cropping window, or manually specify the 'grid' of clones on the microarray.

3 **Computing centers**
Pathways™ automatically computes the centers of the clones on the microarray.

4 **Verifying centers**
After computing the centers, Pathways™ displays the computed points for review and any necessary adjustment.

5 **Writing output**
The import process is complete when the Pathways™ sample and image files are written.

With a description file for a microarray, these Pathways™ files contain information necessary to analyze microarrays in Pathways™ 4.

In **interactive** mode, it is necessary to interact with the import process in Steps 2 and 4. Other steps are automated by Pathways™. In **batch** mode, Pathways™ performs steps automatically. The **Trim** option automatically trims the boundaries before storing the image in Pathways™ image format. Trim reduces the file size of the stored image.

5.2 Supported Image Formats

Phosphor imaging systems store data in image file formats; the data represent intensities for each pixel in the image. The intensity values may be encoded (scaled) to make the image files smaller.

Tiff, Fuji, and Pathways™ image formats are supported in Pathways™ 4. Image readers are **pluggable** in Pathways™, so the program can be extended to include any other image format.

The Tiff option reads grayscale **Tiff images** ('.tif' or '.gel') with or without square root encoding. The Fuji option reads the Fuji Bas scanners' **img/inf** file combination (see Appendix II for details). The **Pathways™ image** format is a proprietary image format that stores raw image data, image encoding parameters, and computed clone center locations after the import process. The format is described in more detail below.

Users of Molecular Dynamics and Packard phosphor imaging systems

Images originating from Molecular Dynamics and Packard brand phosphor imaging systems use a special encoding of the TIFF standard, characterized by a .GEL or .TIF extension. While Pathways™ software is capable of reading these specially encoded files, do not open them in another application first. Opening the images in a graphics application (such as Adobe Photoshop™) strips out critical information used to decode the image, leading to incorrect pixel intensities in the image.

5.3 Microarray Description Plug-Ins

The variety of available microarray products differ in geometrical layout, biological contents, and manufacturing materials (*e. g.*, glass *versus* nylon membranes). The differences in each product potentially require different algorithms to describe the geometry, determine the location of clone centers, and sample the data.

Pathways™ 4 has a generic Microarray Description plug-in option (the GF Description plug-in) that allows any microarray product to interface with the Pathways™ suite of tools. The GF Description plug-in has the following responsibilities.

- **Providing Pathways™ with a description of the product geometry (for example, describing the arrangement of the oligos or clones on the supporting material)**
- **Providing Pathways™ with meta data (accession, description, *et cetera*) for each clone on the microarray**
- **Providing Pathways™ with the location of clone centers on an experimental image**
- **Miscellaneous customizable items**

The GF Description plug-in accesses this file to provide Pathways™ with necessary information for each clone. **These description files can be augmented with additional data**. GeneFilters® description files can be updated through the ResGen™ data server.

The user must select a **microarray brand** (*e. g.*, GeneFilters®) and a **microarray name** (*e. g.*, GF200). When the brand and type are specified, Pathways™ automatically locates the appropri-

ate microarray description plug-in (*e. g.*, 'ResGen™ GeneFilters®').

5.4 Finding the Location of Clone Centers

Once the appropriate image format and description for the microarray is established, Pathways™ searches for clones on the microarray image. Pathways™ has two methods for locating clones on an experimental image: autocentering and template centering.

With autocentering, Pathways™ searches for clones on the experimental image without user input. The autocentering algorithm looks for patterns indicating rows and columns in the image and then focuses on these rows and columns to locate clones. Experimental images are of varying quality, and the autocentering algorithm may not locate all the clones or it may misidentify rows and columns. Therefore, **visually inspect the results of the autocentering algorithm**.

It is possible to give the autocentering algorithm a 'hint' as to where the microarray is located on the experimental image by dragging a cropping rectangle around the microarray. The algorithm finds the clone locations as before, but it does not look outside the cropping window.

The cropping rectangle is available only during interactive importing. In batch mode, it is impossible to specify the cropping rectangle (refer to Chapter 6 for more information about these importing modes).

With template centering, drag a template (a sketch of the microarray layout) on top of the experimental image and adjust alignment points until they match the experimental image exactly. The centering algorithm uses the template to identify the clone locations, rather than searching the entire experimental image.

The most common use of template centering is when the autocentering algorithm fails during importing. In addition, an alternate microarray product might not lend itself to autocentering (for example, when there is no periodic, identifiable pattern to the clone layout). In this case, the alternate microarray product would rely on the template mode for image importing.

5.5 Sampling Microarray Data

When the clone locations on the image are known, Pathways™ samples each clone's intensity and background. In general, the sampled data for each clone includes an intensity and either an overall background intensity or a background intensity per clone. The values of the clone and background intensity are determined with the sampling plug-in to allow sampling algorithms to be added.

5.6 Pathways™ Sample and Image Files

Once the microarray data is sampled, Pathways™ stores the imported data in two files: a Pathways™ sample file and a Pathways™ image file. The **Pathways™ sample file** (‘**.pws**’ extension) contains the sampled clone intensities. The **Pathways™ image file** (‘**.pwf**’ extension) contains the calculated clone locations and a full resolution version of the original image. The image may be rotated and cropped, depending on the alignment of the clones on the original image.

Pathways™ 4 uses the Pathways™ sample file for data analysis. The Pathways™ image file provides **thumbnail** pictures of the currently selected clone in an analysis window. The Pathways™ image format allows rapid file-based access to the raw image data for clones. The advantage of file-based access is that the entire image need not be loaded into memory to view clones, thereby dramatically decreasing the memory requirement for thumbnail views in the analysis modules.

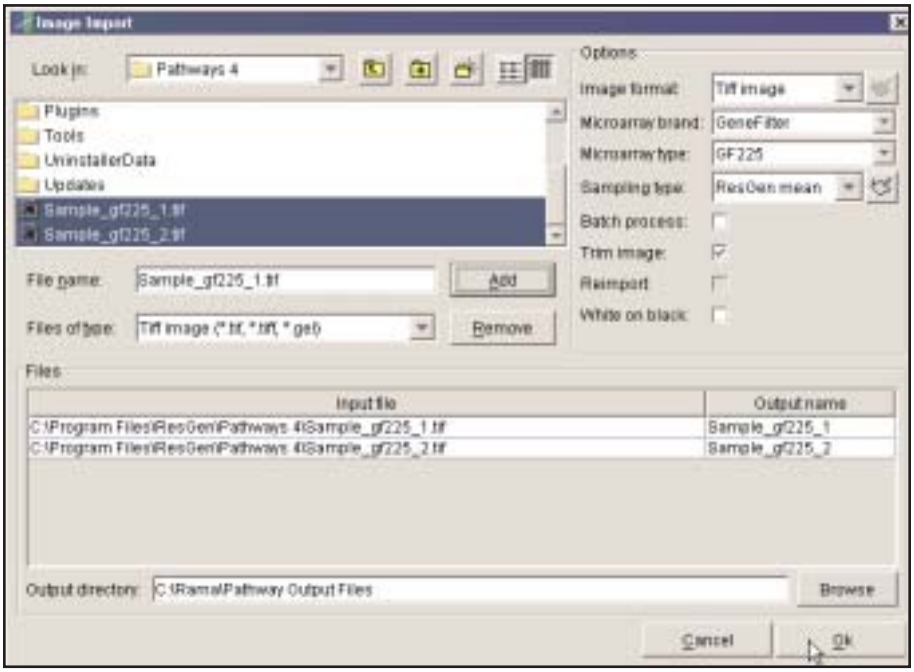
Pathways™ 4 does not require the Pathways™ image file to perform data analysis. If a sample file (pwf) is loaded and there is not an accompanying image file, Pathways™ issues a warning and then proceed with the analysis. The only noticeable difference is that a question mark icon is displayed in place of the selected clone thumbnail. Having two files means that the sample file is small and can be shared between researchers, whereas the image file (pwf), being a full resolution image file, can be 5 MB or larger.

Pathways™ image files can be read back into Pathways™ 4. A Pathways™ image file can be treated as a regular image file with the usual import procedure. In addition, Pathways™ 4 files can be reimported into Pathways™ without locating the clones, because this information is already in the Pathways™ image file. Therefore, it is possible, for example, to change the sampling technique for a file without finding clone centers. Likewise, it is possible to reimport and adjust clone centers (if the center is found to be in error) without performing global alignments.

Chapter 6: Importing

6.1 Image Import Dialog

The image import dialog sets the relevant parameters for an import session. Open the import dialog: **File→Import Image** (CTRL+P)



Pathways™ Universal users may see a completely different import dialog if they are using a Framework that implements importing other than the Pathways Framework.

Before adding images to the import window, adjust the **Files of type** selection to indicate the appropriate image extension. Next assure that the **Image Format**, **Microarray Brand**, **Microarray type**, and **Sampling type** are adjusted appropriately. Check the **Batch process** box to process these imports in a batch mode (see the section on batch importing for more details). Select the **Trim** option to automatically trim the image to reduce the size of the saved Pathways™ image file.

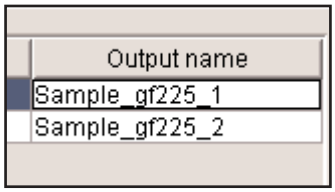
The **Reimport** box is active when the selected image format is a Pathways™ image. A Pathways™ image can be imported as a regular image. Otherwise, skip the initial centering step, and read the computed centers from the Pathways™ file. When reimport is selected, the initial centering step is skipped, and the import process proceeds directly to data sampling and clone adjustment. **This feature is useful if the purpose of the reimport is to adjust a single spot or to resample the data using a different sampling technique.**

Normally, the importer expects to find black spots on a white background. Select the **White on**

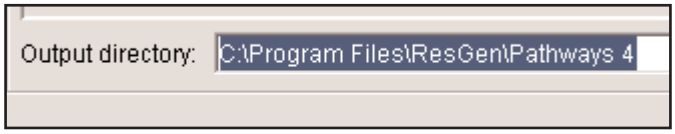
Black option to load the file as a white image on a black background. This option should be selected if raw image file consists of white spots on a black background. The White on Black option works differently from the Contrast Controller Invert option, which takes an image that has already been imported and inverts it, turning the black spots into white spots on a black background.

To add images to the import window, click once on the file to highlight the first import file in the file browser, and click the *Add* button or double click the file icon to the left of the file name. Add multiple files by following the same procedure (they must be of the same microarray brand and type and image format). Remove files from the list by highlighting the desired file(s) and clicking the *Remove* button.

The default output file name for the imported data is the same base name of the input file, but with a different extension. **To change the name of the output files, edit the output name column by double clicking in the selected file table and typing the new file name**.



To change the output directory for the imported files click the *Browse* button next to the output directory field and select a directory from the output directory dialog box. A directory **must** be selected in the directory dialog box for the *Ok* button to register the new directory.



In attempting to import an image with the same output file name and directory as an existing Pathways™ file, a dialog box appears and requests verification before overwriting the existing file.

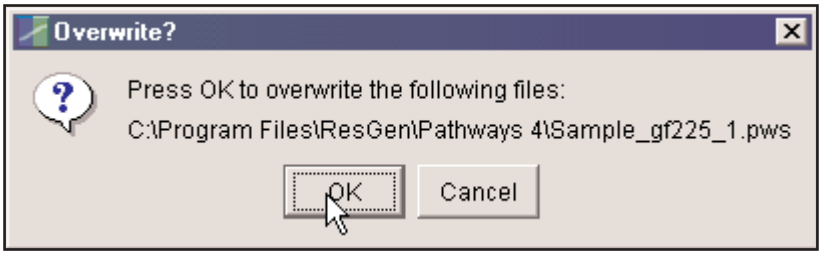


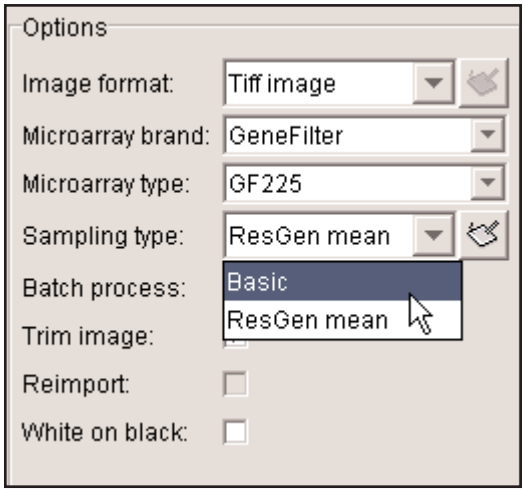
Image format, Microarray brand, Microarray type, and Sampling type are plug-in choices that may have additional, specified properties. As an example, a sampling plug-in might allow

changing the area around each sampled spot. When additional options are present, the button to the right of the plug-in is enabled. Clicking this button allows specification of additional options.

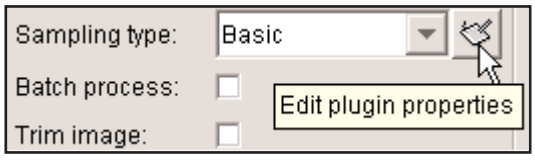
The default **Sampling type** for ResGen™ GeneFilters® microarrays is called **ResGen™ mean**. This algorithm visits each clone center and determines the mean image intensity inside a circular area surrounding the clone. The background level is determined by sampling intensities in the gap between fields 1 and 2 of the GeneFilters® microarray.

The ResGen™ mean sampling routine is set by default to sample 75 % of a spot. A pop up menu next to the sampling selection in the dialog box (discussed below) allows changing the default setting. ResGen™ recommends this default setting. However, if the default sampling area needs to be changed, do not compare imported images with two different sampling areas. If the sampling area size is changed, the new size is remembered in future Pathways™ sessions to help assure consistent sampling from session to session.

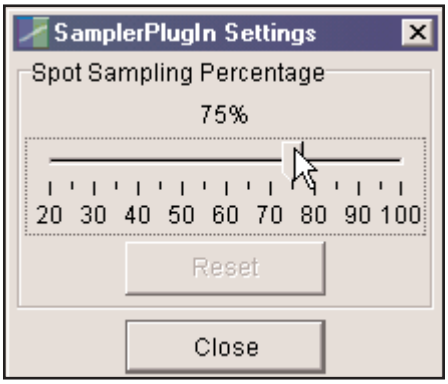
The available sampling routines are dependent on the brand of Microarray (*e.g.* GeneFilters® are the only brand to use the ResGen™ mean). The **Basic** sampling type is available for all microarray brands. To change the sampling routine, select the Basic sampling type.



Click the **Edit plug-in properties** button, located next to the Sampling type field.



The **Sampler plug-in Settings** window appears. The spot sampling percentage may be adjusted by moving the slider bar.



The Basic sampling type is a more generalized version of the ResGen™ mean sampling type that allows the user to adjust the spot sampling percentage. Instead of determining the background level by sampling intensities in the center of the image, the Basic sampling type samples a strip around the edge of the image. The spot sampling percentage is set to 75%, but the user should experiment with different sampling percentages for different microarray types.

6.2 Introduction to Interactive Importing

Interactive importing is launched after clicking the *Ok* button in the Import dialog box without the Batch option checked. Interactive importing consists of five steps for each image.

1 Load image

2 Align / Crop / Template

3 Compute centers

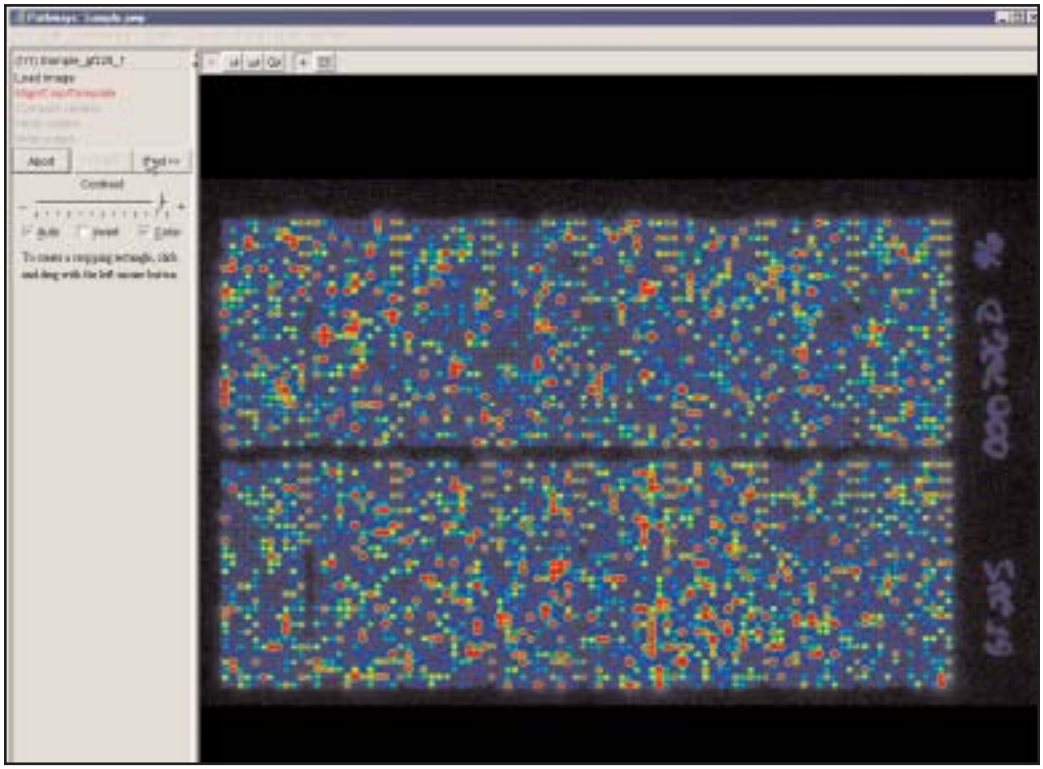
4 Verify centers

5 Write output

Interaction with the import process is required for only Steps 2 and 4; Pathways™ performs other steps automatically. The import window shows the current step highlighted in the upper left corner.

Contrast slider and color options are available throughout the import process to enhance the displayed image. **The contrast and color have no effect on the data sampling**; instead, they enhance the displayed image. Traditional gray scale images are displayed in 256 shades of gray. False coloring makes 256 values available for each of the color channels (red, green, and blue), and it can display over 16 million colors for the same image. See the graphical interface overview chapter for instructions on adjusting the image contrast.

The first window that appears is the image alignment window.



The image alignment window allows rotating the image and optionally applying a cropping rectangle (auto / crop mode) or template overlay (template mode). The Auto / Crop mode invokes the fully automated centering routine, while the template mode uses a template overlay to locate the centers.

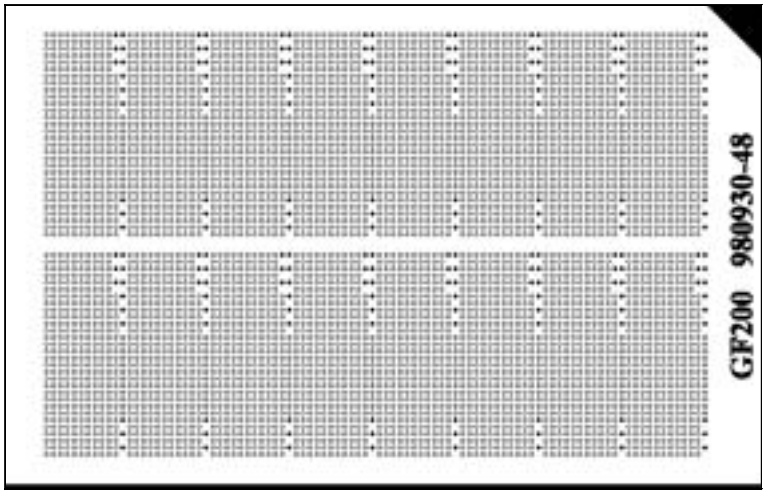
Use the four rotation buttons to rotate the image by 0, 90, 180, and 270 degrees, respectively.

Rotation allows the image to be aligned.

Array images created using the Array Designer must be rotated to correspond with the same image orientation used in the Array Designer.

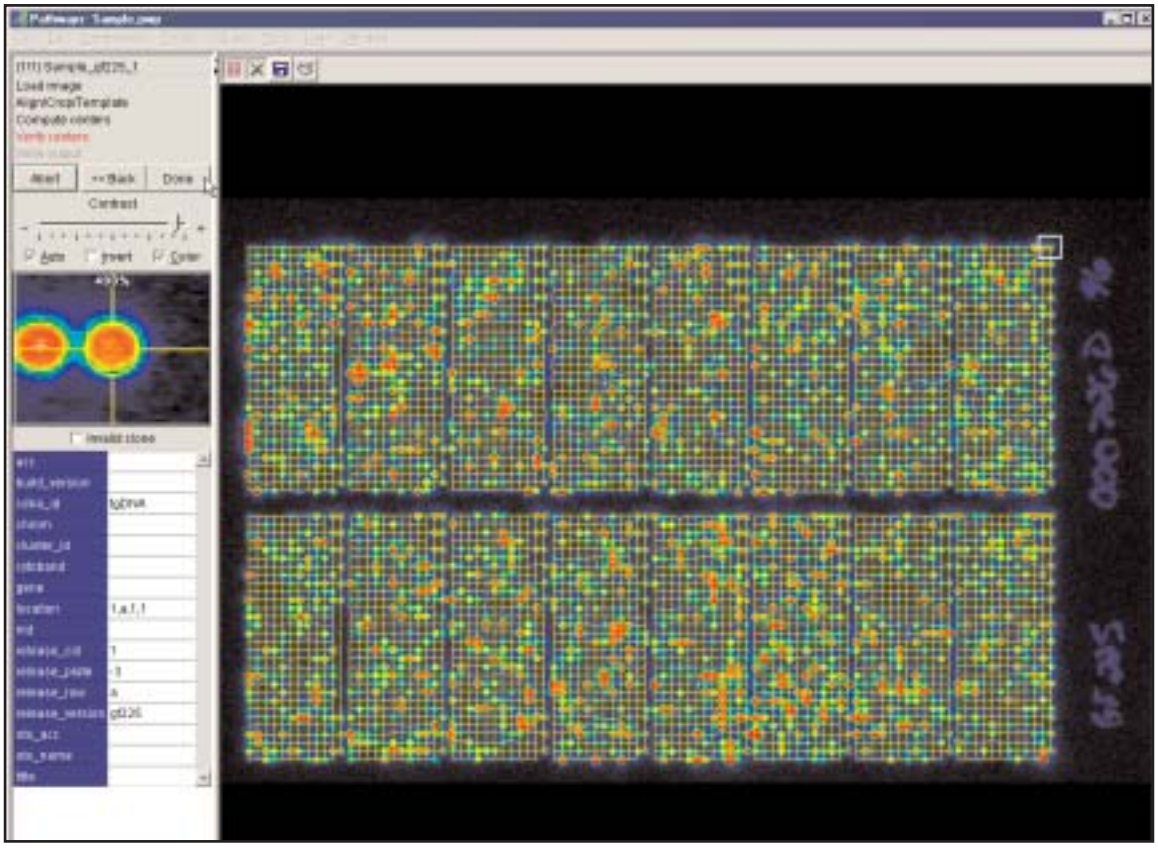
ResGen™ GeneFilters® Microarrays Users

Most GeneFilters® images autocenter without problems (assuming the orientation is as shown below). In general, first try the autocentering without a cropping rectangle (click *Next* in the alignment window). If Pathways™ cannot find all the clones in this mode, add a cropping rectangle. If Pathways™ still cannot find all the centers, use the template mode.



After the initial alignment setup, click the *Next* button; Pathways™ calculates the clone centers. When Pathways™ is unable to calculate the clone centers, the alignment window is redisplayed, and there is a prompt to adjust the alignment.

On successful alignment, the alignment verification window appears.



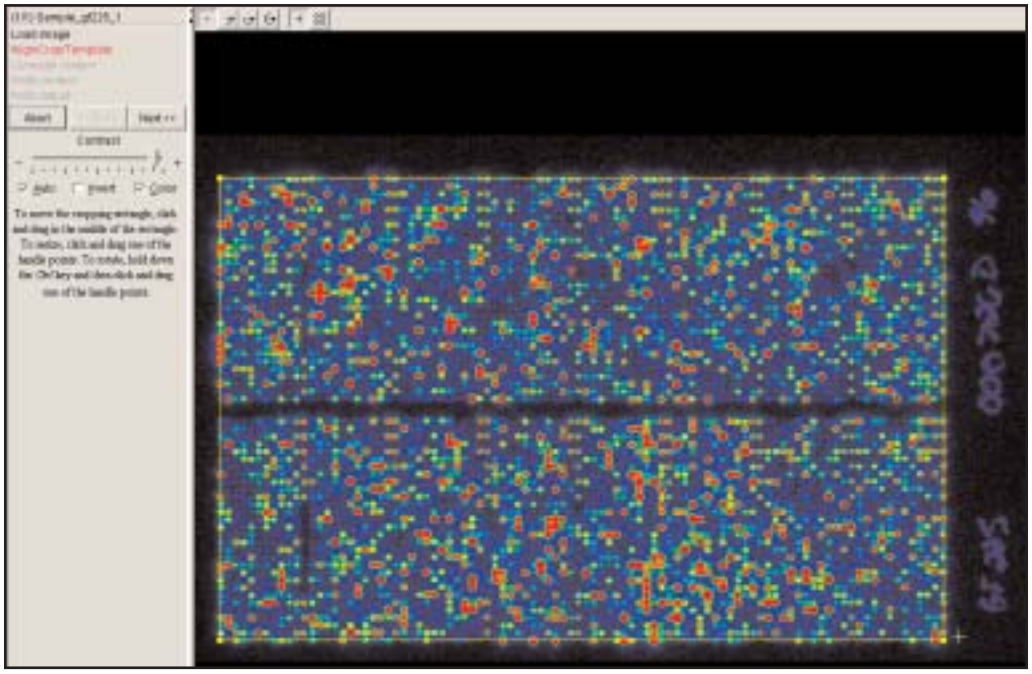
When the centering process is not satisfactory, click the *Back* button to revert to the previous window and adjust the centering parameters (see discussion below on cropping rectangles and templates). Click *Done* to write the Pathways™ sample data and image files and to complete the import process. The following sections describe the interactive importing windows in more detail.

6.3 Interactive Importing: Auto / Crop Mode

The auto / crop alignment mode invokes an automated algorithm that finds clones in the image. A cropping rectangle can be dragged over the image to better define where the arrayed spots are located. The **crop / auto** and **template** buttons are located to the right of the rotation buttons.

Use a cropping rectangle when multiple microarrays are within the image or when Pathways™ cannot find all the centers in the fully automatic mode.

The cropping rectangle lies outside of the arrayed spots without overlapping the spots.



Perform the following operations to use a cropping rectangle:

- **Click anywhere on the image and drag for the cropping rectangle to appear.**
- **Click anywhere inside the cropping rectangle and drag to move it.**
- **To stretch the cropping rectangle drag any of the handle points on the side or corners of the rectangle.**
- **To rotate the cropping rectangle, press the Ctrl key, and drag any of the corners (the center of rotation is the opposing corner).**
- **To remove cropping rectangle, click anywhere outside the rectangle.**

When satisfied with the image orientation and with the positioning of the cropping rectangle (if used) click the *Next* button to invoke the Pathways™ autocentering routine.

6.4 Interactive Importing: Template Mode

The template mode allows more precise specification of the alignment of the arrayed spots in the image. The template mode overlays a template (skeleton representation) of the microarray layout on top of the experimental image. When the template is lined up properly with the experimental image, the clone centers can be determined by referring to the manufacturing lay-out of the microarray.

The template mode involves the following two stages.

1 **Global alignment**

2 **Adjusting alignment points (with or without magnifier)**

The **Global** setting drags (and rotates and stretches) a global rectangle that has the microarray template type attached to it. The function is similar to that of the cropping mode discussed above, but **the objective is to use the global rectangle to align the template on top of the microarray data, rather than to place the rectangle outside the microarray data points**.

After the templates are adjusted using the global rectangle, the location of the **alignment points** can be further refined using fine adjustments to alignment points in the template. These points can be adjusted either with or without a magnifier, a feature that locally enhances the image for more accurate adjustment of the alignment points.

Template mode is selected using the **template** button at the top of the import window.

Adjust the global setting.

Select Adjust Global (initially selected by default).

Drag / Resize / Move / Rotate the global rectangle as with a cropping rectangle (instructions above).

As the global rectangle is dragged, a **template** is attached to it. Use the global rectangle as with a cropping rectangle to align the template on top of the experimental image.

After the global alignment, uncheck the **Adjust Global** box, and refine the location of the template alignment points.

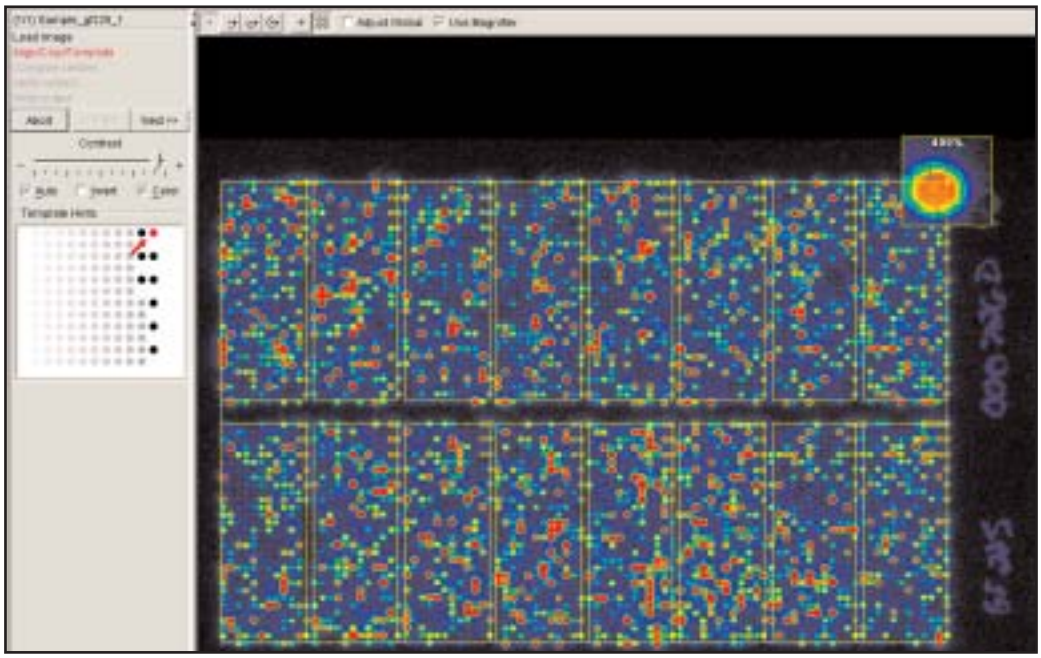
To adjust template points with a magnifier present, perform the following steps.

1 **Deselect the Adjust Global option, and select Use Magnifier.**

2 **Move the pointer over each alignment point, and a magnifier window appears (an alignment hint picture also appears on the left panel).**

3 **Click anywhere in the magnifier window, and the alignment point snaps to this location.**

4 **The magnification can be increased or decreased using the up or down arrow keys while the magnifier is present.**



Perform the following steps to adjust alignment points without magnification:.

Deselect the Use Magnifier check box.

Click and drag the alignment point to move it.

With ResGen™ GeneFilters® microarrays, the template alignment points are located over 16 of the control spots for each microarray. In Field 1 of the GeneFilters® microarray, the template alignment points are the upper right control spot in each grid. In Field 2 of the GeneFilters® microarray, the template alignment points are the lower right control spot in each grid (refer to Appendix I). When the control spots are not visible (*e. g.*, in cross-species hybridization), then the alignment points must be in the approximate position where the control spots normally reside; verify the accuracy of the alignment by viewing the overall template positioning.

When dragging a template over a ResGen™ GeneFilters® microarray image, first click the upper right control point and then drag the template to the lower left corner of the microarray. Perform refinements (resizing and rotation) to the global template position with the lower left corner of the global template once the upper right control point is positioned.

6.5 Reviewing Alignments and Saving

The second screen in the import process is the verification window. This window displays the imported image with an overlaid grid representing the detected clone center locations. A detail viewer is shown in the left panel.

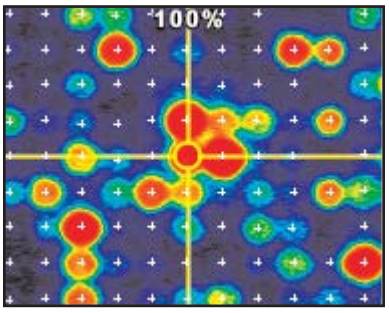
A tool palette at the top of the window allows toggling the grid on or off, displaying of x's to mark invalid clones, exporting the sampled data to a spreadsheet file (comma separated text file), or annotating the image with the researcher name and comments.

To bring clones into focus in the detail viewer:

Click the image at the appropriate location or

Use the up, down, left or right arrow keys

The detail window on the left of the screen can be used to zoom in and out of the image to better review the overall alignment of the image (**up arrow key** zooms in and **down arrow key** zooms out when the cursor is in the detail viewer).



If the overall alignment of the image appears to be inaccurate, click the *Back* button, and repeat the initial alignment step. When repeating the alignment step, add a cropping window or overlay a template for images that are problematic. If clone alignment appears to be inaccurate, then

this clone can be adjusted individually.

To adjust clones, perform the following steps.

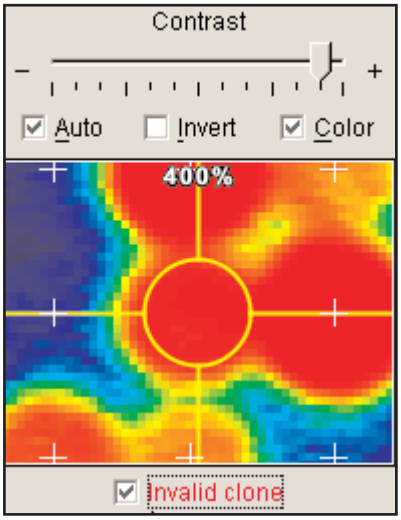
1 **Select the clone (so that the appropriate clone is in the detail window).**

2 **Press the Ctrl key, and drag the alignment circle in the detail viewer.**

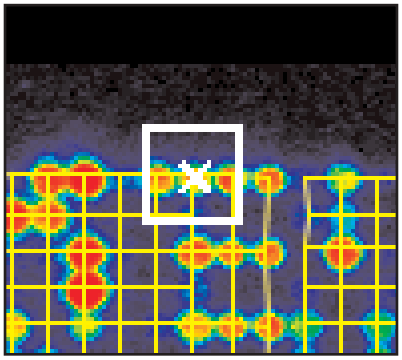
Click the *Done* button to complete image import. When additional images are specified in the import dialog box, the next image loads automatically. When no additional images were specified, the import process stops.

6.6 Invalidating a Clone

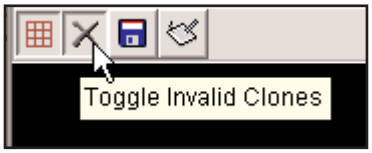
For microarrays originating from an image, a spot can be marked as invalid during the image import process, after spot centers have been identified, by checking the **Invalid clone** box below the detailed view of a selected spot.



Invalid spots are marked on the main microarray view with crosses.



Such markings can be shown or hidden by selecting and deselecting the **Toggle Invalid Clones** button on the toolbar in the upper left corner of the main view.



Spots marked as invalid during the image import process are seen as such initially during future uses of the resulting microarray file in projects.

Refer to Chapter 9 for more information about Invalid Clones.

If analyzed microarray data comes from a spreadsheet file, clones with missing intensity or background data are marked as invalid automatically.

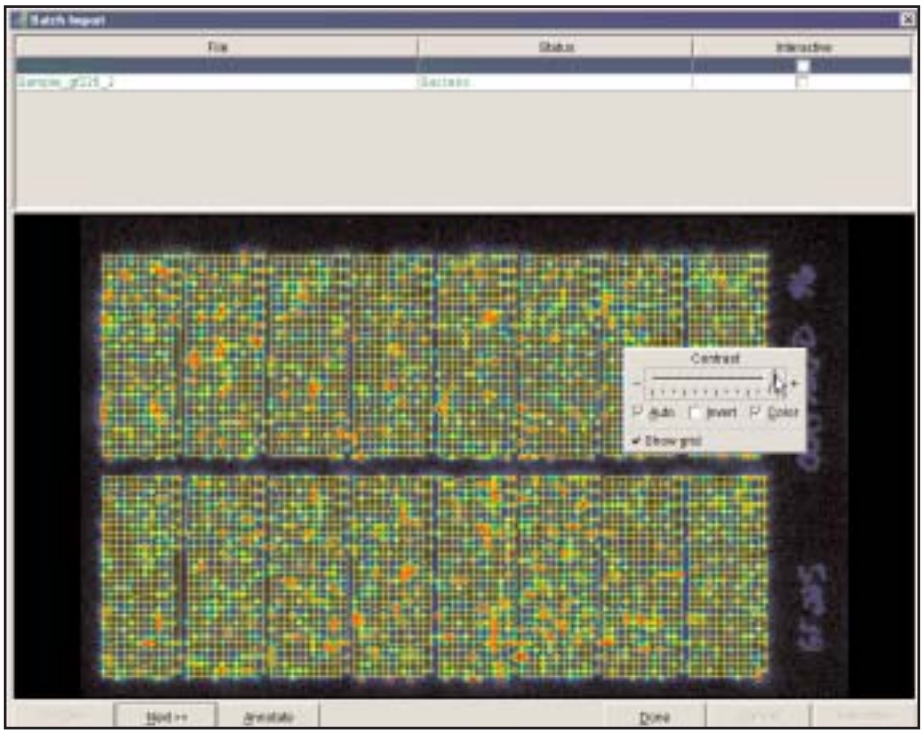
6.7 Batch Importing

Batch processing allows for the rapid, automatic import of multiple images. From an algorithmic standpoint, batch processing mode is identical to interactive mode without applying the cropping rectangle. The autocentering algorithms in Pathways™ 4 are robust. However, the quality of experimental images varies, and the autocentering algorithm can fail to locate all the clones or incorrectly identify rows and columns, especially for images with relatively low signal levels (even if they appear to be normal by visual inspection). Therefore, **visually inspect the results of the autocentering algorithm**. To determine how well the autocentering algorithms work on experimental images before proceeding to a full batch mode, import multiple experimental images in the iterative auto / crop mode.

To start Batch importing, select **Batch Process** in the import dialog box, and click the *OK* button.

The batch import dialog box appears and shows a table of files being imported and the status of each file in the import process. This dialog box begins the import process for images that were selected in the import dialog box. When the import process is completed, the status for images is listed in the second column. If the autocentering algorithm does not locate all the clones in an image, then the **Interactive** box is checked next to the image, indicating that Pathways™ proceeds with an interactive importing session for this image.

When the files have been imported, the images and alignments can be previewed either in a slide show fashion by successively clicking the *Next* or *Previous* buttons or by selecting files from the table.



Annotation information (Researcher / Comments) can be added by using the *Annotate* button.

The image contrast and color scheme can be adjusted by a menu that pops up after right clicking on the image (see the section on image contrasting in Chapter 2). The grid can also be turned on and off through this menu.

If an alignment passes autocentering but it still does not appear to be correct, select the check-box to interactively import the image.

After previewing imports:

If one or more images are checked for interactive import, click the *Interactive* button at the bottom of the dialog box to proceed with interactive import for the selected image(s)

or

Click the *Done* button. If imports were successful, it ends the importing process. When any images are marked for interactive importing, a warning is issued before closing the window.

Book IV: Core Concepts

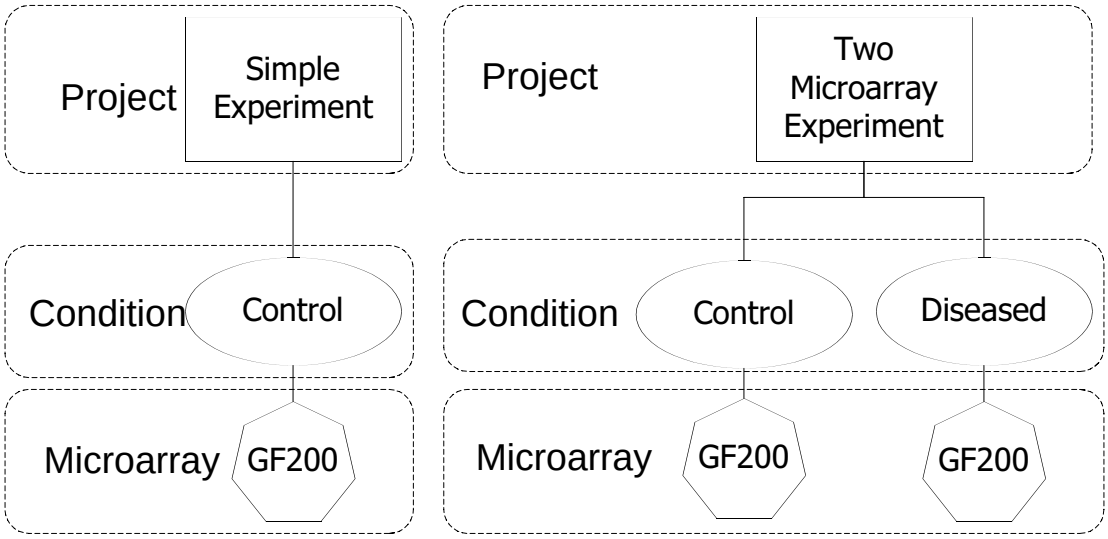
Pathways™ Data Organization and Management

Chapter 7: Pathways™ Projects

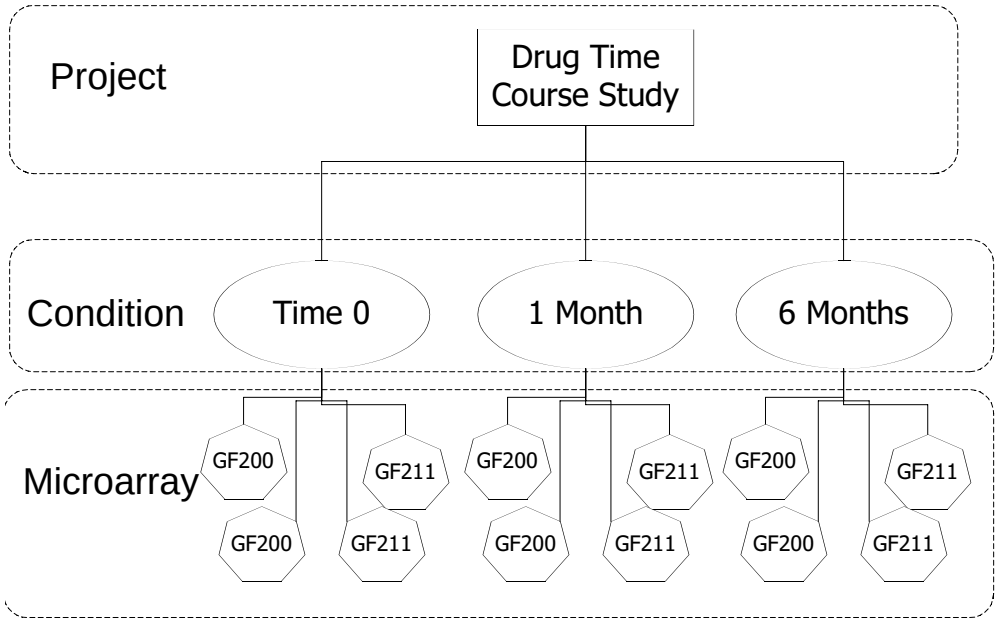
7.1 Projects

A Pathways™ analysis session begins with the creation of a **project**. Pathways™ projects organize previously imported microarray data into **conditions** that represent states in an experiment. Each condition contains one or more sampled microarray data sets.

A simple project can consist of one or two microarrays.



A more complex project can consist of multiple conditions, each containing multiple microarray types and / or repeat data for the same microarray type.



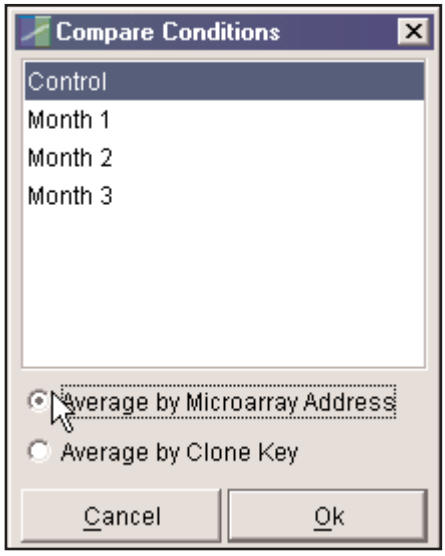
7.2 Conditions

Conditions can represent any experimental grouping. The most common uses of conditions are for state comparison (*e. g.*, normal versus diseased) or for time series comparison (each condition represents a time in the study). Each project can contain as many conditions as are necessary to represent the study.

Microarrays of a single condition need not be of the same type. Instead of analyzing data sets one microarray type at a time (which could, for example, limit the analysis to approximately 5,000 clones for GeneFilters®), the data set can comprise multiple microarray types, enabling analysis of an unlimited number of clones.

Repeated clones in a condition are detected automatically during analysis. If a condition contains repeat clones, then the analysis uses the mean of the normalized intensity for each set of repeated clones. In addition, the existence of repeat elements enables the calculation of experimental statistics for the sampled data sets.

Pathways™ searches for repeat elements based on one of two entered methods: microarray address or clone key.



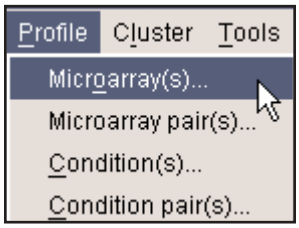
The **microarray address** method identifies repeats based on common microarray types (physical location). For example, when two GF200 GeneFilters® are present in a condition, and the microarray address method is selected, the first clone in the first GF200 microarray is averaged with the first clone of the second GF200, the second clone in the first microarray with the second clone in the second microarray, *et cetera*; standard deviation values for the repeated clones are calculated for use in the analysis process. **This type of averaging does not average multiply spotted clones in a microarray** (if a clone appears in multiple locations in a microarray, the clone key is the same, but the microarray address is different).

A **clone key** is a unique identification that is present for each clone in a microarray. The clone key is usually the accession number of the clone although the key could follow other naming conventions for biological materials that are not in the public databases (for example, ResGen™ uses the string "tgDNA" to identify total genomic spots). The option to identify repeats by clone key searches for repeats of a clone key and groups these together. **Averaging by clone key enables statistical analysis for repeated clones in a single microarray and also enables statistical analysis of the same clone across different microarray types**.

When it is undesirable to average repeated experimental results, place the microarrays in different conditions.

7.3 Grouping of Data

There are four ways of grouping data in a new analysis window: by Microarray(s), by Microarray pair(s), by Condition(s), and by Condition pair(s).



Microarray grouping examines a single microarray to analyze normalized intensities (expression levels). Depending on the analysis type, synthetic arrays, line plots, tables, and other techniques view the data. When multiple microarrays are selected in a comparison analysis, then the data from the microarrays are overlaid (synthetic microarrays and tables appear in tabbed windows, while scatter plots show different colors or symbol types). Profiling and clustering use multiple microarrays to analyze data over a range of experiments.

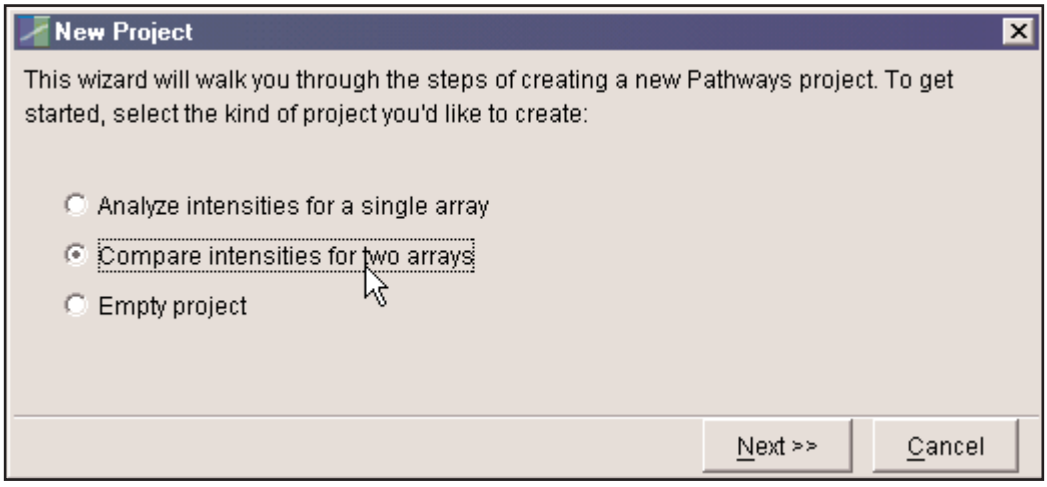
Microarray pair grouping examines the ratios and differences of normalized intensities between a pair of microarrays (differential expression). These data may be viewed in a fashion similar to that of the non-paired option. If multiple microarray pairs in a comparison analysis are selected, then the paired data is overlaid in the analysis windows. Profiling and clustering use multiple microarray pairs to analyze upregulation or downregulation over a range of experiments.

Condition grouping is similar to single microarray grouping, except that the analysis uses conditions rather than microarrays. Therefore, this analysis looks at a condition's average normalized intensity (for repeated clones) across the microarrays. Multiple conditions may be selected to overlay the data (comparison analysis) or analyze the data over a range of experiments (profiling and clustering analysis).

Condition pair grouping is similar to microarray pair grouping, except that the ratios and differences represent the differences or ratios between averaged normalized intensities across the microarrays. Multiple condition pairs may be selected to overlay the data (comparison analysis) or to analyze the data over a range of experiments (profiling and clustering analysis).

7.4 Creating Projects in Pathways™

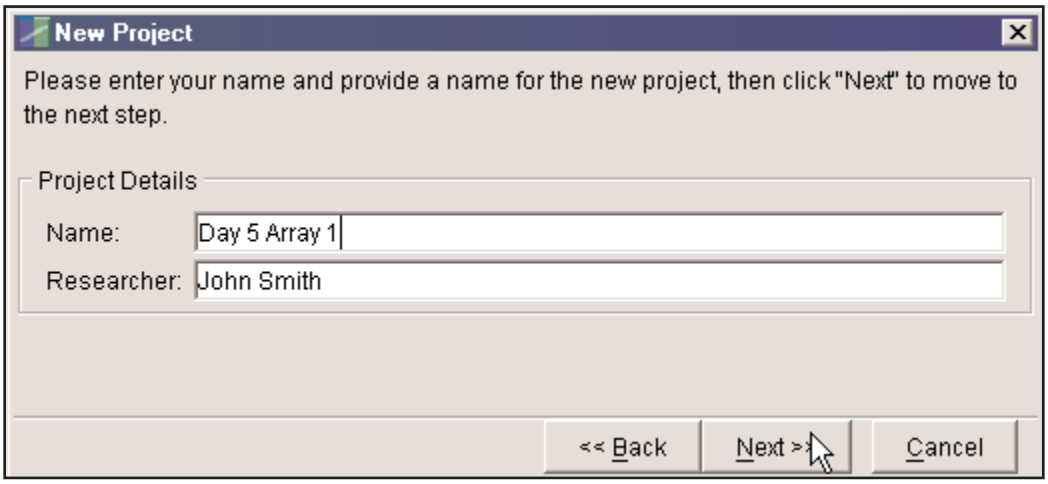
Create a new project in Pathways™ 4 by selecting New Project from the File menu. This selection launches the **Project wizard**, which presents a guide for the creation of new projects. In the first panel of the wizard, there is a prompt to select a single microarray project, a two microarray comparison project, or a new blank project.


New Project
This wizard will walk you through the steps of creating a new Pathways project. To get started, select the kind of project you'd like to create:
Analyze intensities for a single array
Compare intensities for two arrays
Empty project
Next >>
Cancel

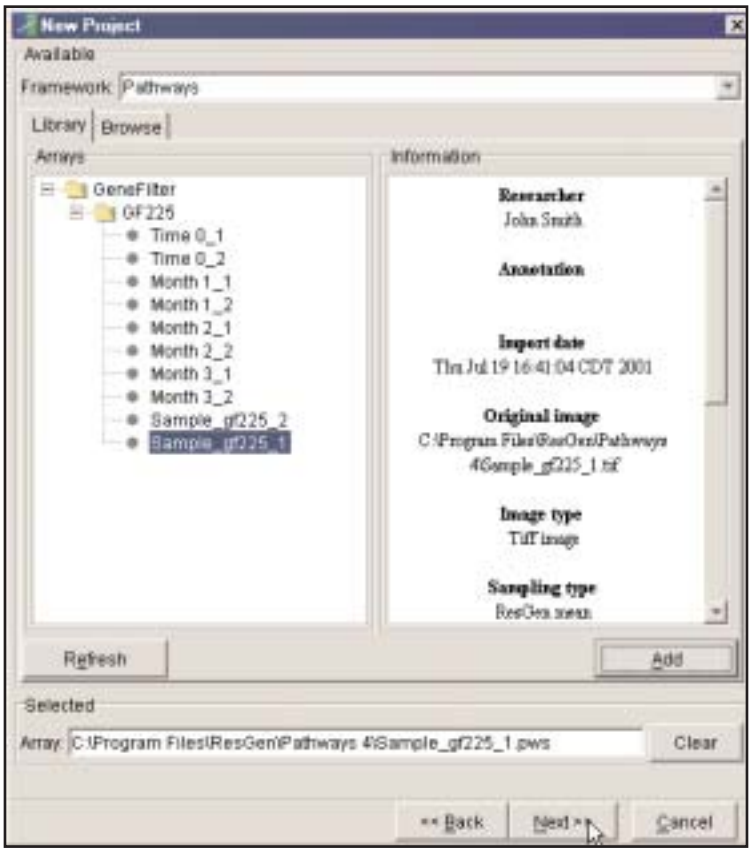
Each of these options is discussed in this chapter.

7.5 Single Microarray Projects

A **single microarray project** is a quick route to analyze normalized intensities for a single microarray. After selecting this option, click the *Next* button. The wizard prompts input for the project and the researcher's name.

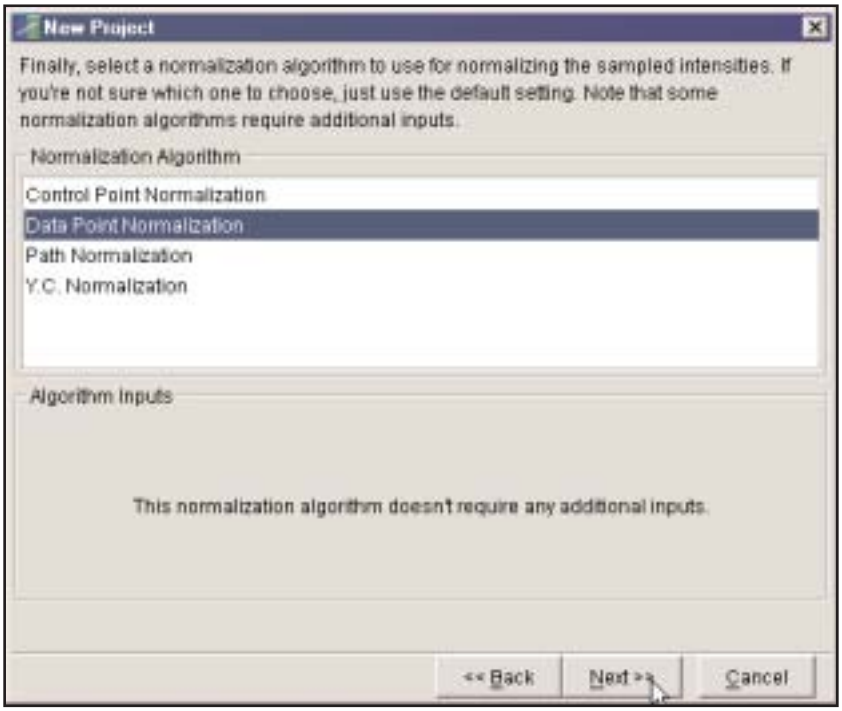


In the next window, a prompt appears for the selection of a microarray for the project.

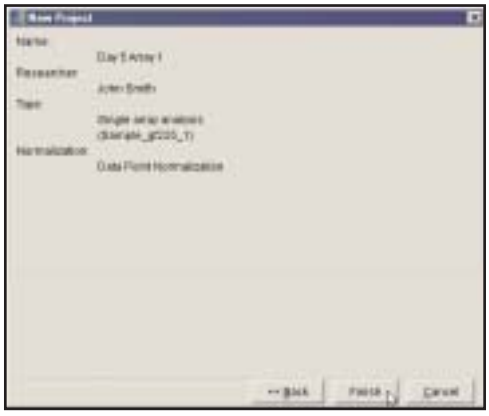


The **Framework** field at the top of the screen allows Pathways™ Universal users to select the appropriate framework from which to obtain the microarray data (refer to Chapter 3 for more detail on frameworks).

Click on a microarray, and then click *Next*. A prompt appears for a normalization technique (use the default value, or refer to the Normalization Chapter for more details).


New Project
Finally, select a normalization algorithm to use for normalizing the sampled intensities. If you're not sure which one to choose, just use the default setting. Note that some normalization algorithms require additional inputs.
Normalization Algorithm
Control Point Normalization
Data Point Normalization
Path Normalization
Y.C. Normalization
Algorithm Inputs
This normalization algorithm doesn't require any additional inputs.
<< Back
Next >>
Cancel

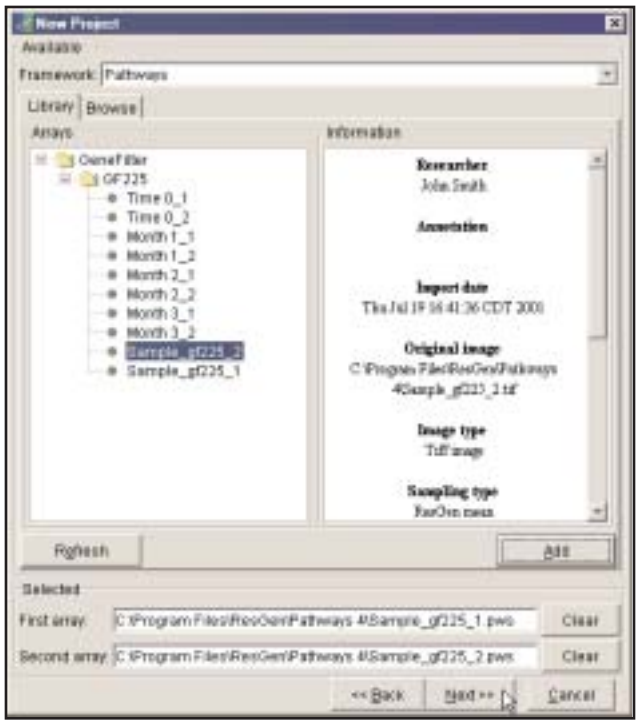
Click *Next* to generate a summary of the project setup.



If items require modification, click *Back*, and make necessary modifications. Clicking *Finish* creates a single microarray project and opens a comparison analysis window.

7.6 Two Microarray Comparison Projects

A **two microarray comparison** project offers a mechanism for generating a project that compares two microarrays. The Project Wizard steps for the two microarray comparison project are identical to those for the single microarray except for the microarray selection page.



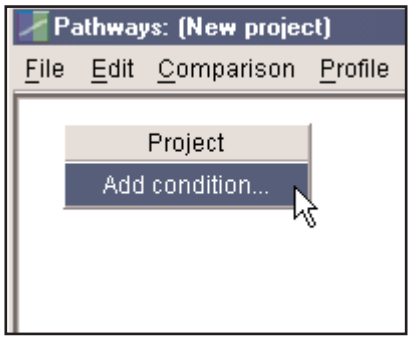
Each microarray for the comparison is selected by clicking on the desired microarray and then clicking the *Add* button for the first array. A second array can be selected similarly. If two arrays are added and the *Clear* button is clicked, the second array will automatically move to the first slot.

Once the wizard setup is complete, a two microarray project is created and a comparison analysis window is displayed.

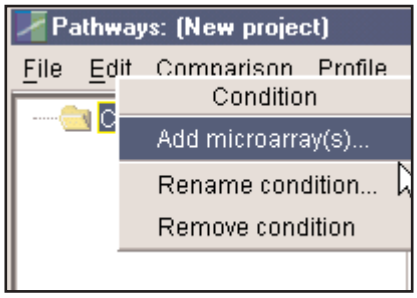
7.7 Empty Project

The **empty project** option in the new project wizard creates a project without any microarrays or conditions. This option is used when setting up more complex projects that have multiple conditions and / or multiple microarrays in each condition. A wizard prompts the user to enter the researcher and project name and creates an empty project.

To add conditions to the project, select **Add Condition** from the **Edit** menu, or right click in the project window outside any conditions or microarrays that have been added.

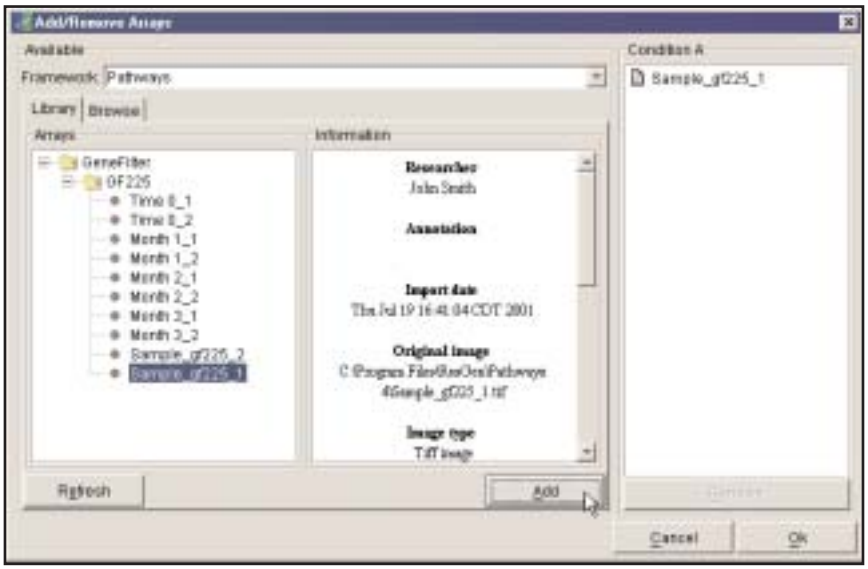


To add a microarray to a condition, either select the condition, and select Add Microarray from the Edit menu, or right click on a condition in the project tree.

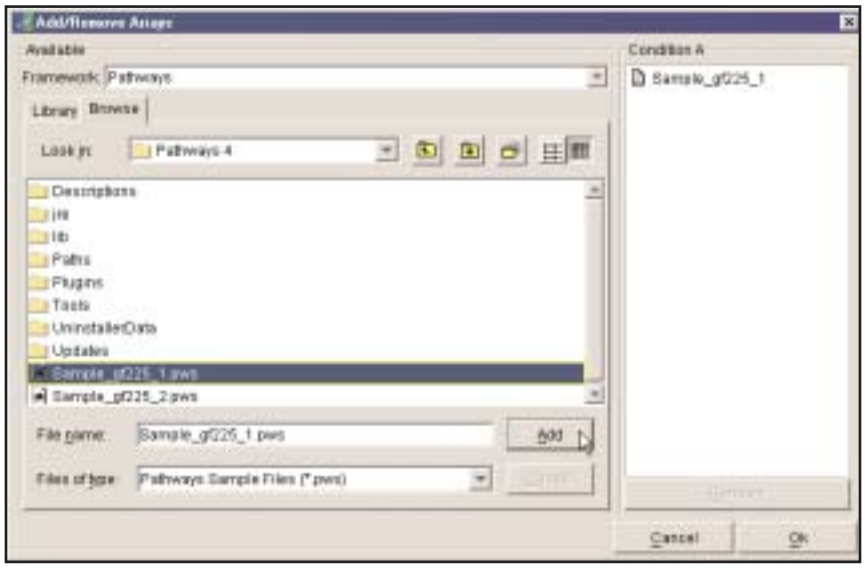


Selecting the **Add microarray** option causes the **Add/Remove Arrays** window to appear. For the Pathways™ Framework, this window has two tabs (for a full description of Frameworks, refer to Chapter 3). The first tabs allows adding at least one microarray to the current condition, based on records in the library file. This file contains information on imported images and / or information on files that have been used in a Pathways™ project. The second panel allows browsing the file system to locate additional Pathways™ files.

Adding microarrays with the Library tab



Adding microarrays with the Browse tab



Clicking on a microarray in either section of the dialog box activates the *Add* button in the dialog box. To add a microarray to the condition, click the *Add* button, or double click on the microarray. To remove a microarray from the Condition, select the microarray in the condition field, and click *Remove*.

Clicking the *Refresh* button in the library section of the dialog box verifies that each file in the library still exists and that the library reflects each file's correct microarray type.

In addition to the basic project creation options discussed above, the Edit menu allows the following modifications.

- **Changing project properties (researcher name, project title)**
- **Editing normalization options (refer to the Normalization chapter)**
- **Renaming or removing conditions in the project**
- **Removing microarrays from the project. Removing conditions or microarrays from the project may affect open analysis windows. In this case, Pathways™ requires closing the affected analysis windows before the Pathways™ session proceeds.**

Chapter 8: Normalization

8.1 Basic Concepts in Intensity Normalization

Microarray experiments may yield sampled clone intensities that are brighter or darker than similar intensities for a reference image(s) (due to experimental variations such as pipetting, hybridization time, *et cetera*). To compare experiments, adjust for global shifts in intensity levels, so that, for example, high intensity ratios truly represent upregulated genes.

Normalization algorithms correct for global intensity shifts across multiple experimental images. The normalization process creates a set of 'normalized intensities,' which vary from the sampled intensities by a scaling factor. These normalized intensities are the basis for Pathways™ 4 data analysis.

8.2 Pathways™ Normalization Algorithms

Four standard normalization plug-ins are included with the basic Pathways™ 4 installation. In addition, normalization is a **pluggable** component and therefore new normalization algorithms can be added to Pathways™ at any time.

Data point normalization is the default normalization technique for Pathways™ 4 GeneFilters® microarray analysis. This technique generates normalized intensities by dividing sampled intensities by the mean sampled intensity of all clones, **except the control points, total genome spots identified by the key tgDNA**.

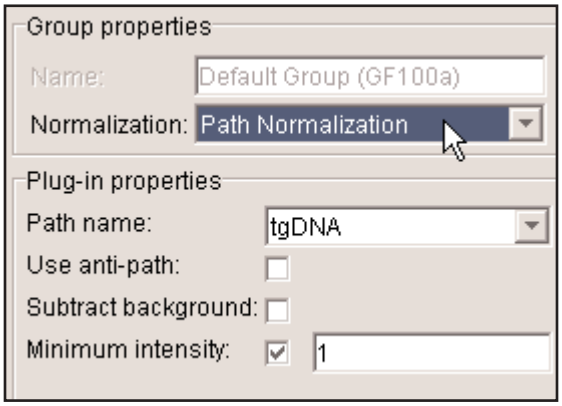
Pathways™ 2 users

Pathways™ 2 normalized spots such that the average normalized intensity for spots was 2,000 (based on empirical observations of microarray intensities). Pathways™ 4 normalization typically normalizes the intensity to an average of 1.0. This normalization allows the Pathways™ 4 user to immediately recognize normalized values that are greater than the mean sample (> 1.0) or less than the mean sample (< 1.0). To compare Pathways™ 4 normalized intensity values directly with Pathways™ 2 normalized values, divide Pathways™ 2 normalized intensity values by 2,000.

Control point normalization normalizes sampled intensities by dividing each sampled intensity by the mean sampled intensity of the control points present on the current microarray.

Path normalization is a normalization technique that normalizes sampled intensities by dividing each sampled intensity by the mean sampled intensity of a defined criterion (path). With GeneFilters®, Control Point Normalization is an example of Path normalization (the path selects "tgDNA" spots). When different types of microarrays are grouped together for normalization,

only the elements of the path that are common to all microarrays in the group are used in the normalization process.



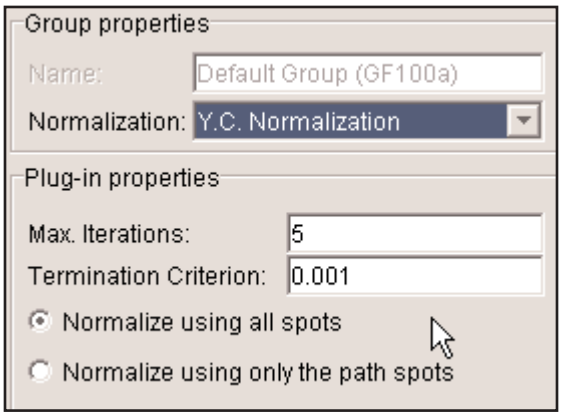
Path normalization has a checkbox selection labeled **Use anti-path**. An anti-path represents all spots except those contained in the path. Anti-path normalization therefore normalizes the data, using points that are not contained in the path. With GeneFilters®, Data Point Normalization is an example of a Path normalization technique in which the 'clones not in path' option was selected (normalize by points **not** in the "tgDNA" path).

Path normalization also has a checkbox selection labeled **Subtract background**. When this box is checked, the input to the normalization algorithm will be the difference between the intensity and the background intensity (intensity - background intensity) for each spot, rather than intensity alone.

A **Minimum intensity** level for the normalization algorithm may also be specified. This value will be applied either to the intensity alone or to the difference between the intensity and the background intensity if background subtraction is enabled. The minimum intensity value is typically used to assure that all inputs to the normalization algorithm are greater than zero. If zero or negative intensity values are present in the normalized data sets, then ratios will be disabled for microarray pair and condition pair analysis. Pathways disables these ratios to prevent division by zero errors and ambiguous ratio values (*e.g.* a ratio of -1 could result from an intensity of 1 in the first spot and -1 in the second spot).

Y. C. normalization is a normalization technique adapted from a manuscript by Yidong Chen *et al*. (Chen Y, *et al*., *J. Biomedical Optics* **2**(4): 364-374, October 1997, ISBN 1083-3668). This manuscript covers several important topics in microarray analysis, including an iterative normalization technique that normalizes a pair of microarrays such that the mean **ratio** between the microarrays is 1.0. A number of assumptions derive the normalization technique; review the manuscript before using this technique.

As the technique is iterative, it does require as input the maximum number of iterations (**Max. Iterations**) and a **Termination Criterion**. Five iterations are sufficient to ensure that the mean ratio between the arrays is 1.0, although it is possible to select a larger number of iterations. The algorithm stops the iteration process if the difference between the calculated mean ratio and 1.0 is less than the termination criteria.



The normalization technique has been extended to normalize more than two microarrays by setting the first microarray in a multiple microarray set as the reference microarray and normalizing the ratios of the other microarrays to the first microarray. The user can select **Normalize using all spots** to do this. The concept of paths has been added to this algorithm, so that the mean ratio can be estimated using only the spots in a path (or outside of a path, depending on the selected option). The user can select **Normalize using only the path spots** to do this.

This normalization technique does require that microarrays in a normalization group (see below) are of the same microarray type.

8.3 Normalization Groups

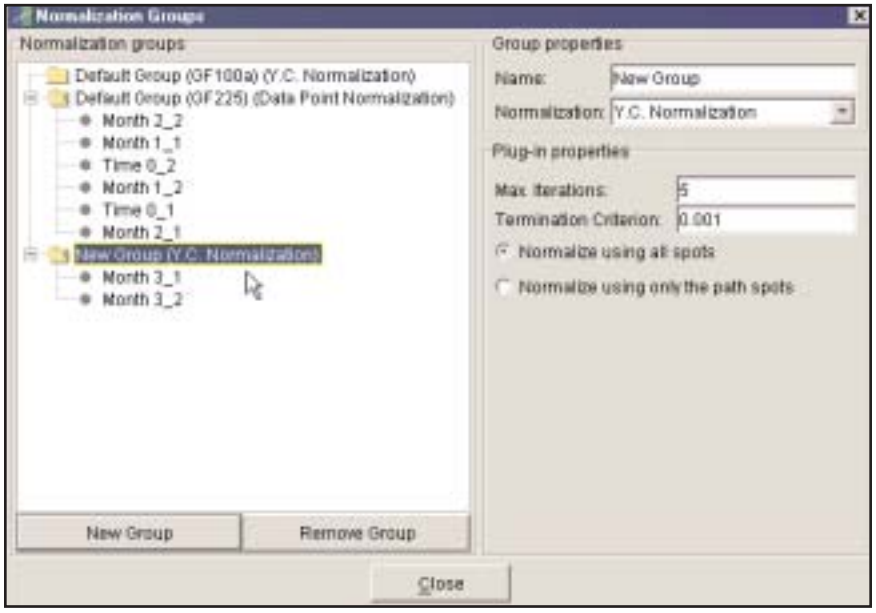
Normalization techniques generally fall into two classes: auto normalization and dependent normalization. An **auto normalization** technique normalizes intensities based on data that are contained completely in the microarray data set. For example, the Data Point Normalization technique is performed by dividing sampled intensities in a microarray by the mean sampled intensity value in the same microarray.

Dependent normalization techniques exhibit a dependency on other microarrays to perform the normalization algorithm. The Y. C. Normalization algorithm, for example, normalizes a pair of microarrays to the mean ratio of intensities between the arrays. The Path Normalization algorithm is structured so that only the elements of the path that are common to all microarrays are used in the normalization algorithm.

Pathways™' **normalization groups** explicitly assign a normalization technique to a group or groups of microarrays. These groups enable the following options.

- **Assigning different normalization techniques to subgroups of microarrays in a project**
- **Applying the same normalization technique to different groupings of microarrays in a project (used to selectively apply dependent normalization techniques)**

By default, Pathways™ 4 groups microarrays by their type (*e. g.*, 'GF200', 'GF211'), and it applies the data point normalization technique (mean of all points, except controls). To change normalization techniques and / or normalization groupings, select 'normalization' from the project menu to generate the following dialog box.



The window shown above was created from a project that had only one microarray type, GF200. As discussed above, initially all of the GF200s were grouped into a default group with data point normalization. To change the normalization technique, select this group in the **Normalization Groups** window, and edit the group properties. In addition to the group properties, each normalization plug-in may require additional data items (*e. g.*, maximum iterations for Y. C. Normalization), which appear in the plug-in properties window.

To add a new normalization group, click *New Group*, and edit the properties. Individual microarrays can be moved between normalization groups by dragging them from their current folder and dropping them into a new folder. Normalization groups can be removed by highlighting the group and clicking the *Remove group* button.

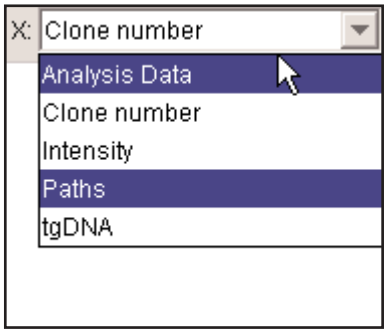
Chapter 9: Data, Paths, and Filters

9.1 Analysis Data

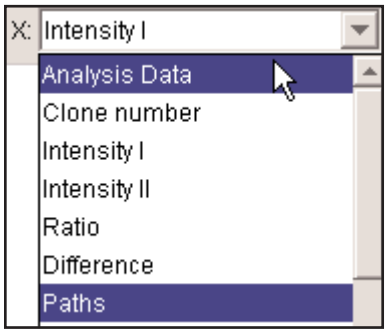
Pathways™ supports a set of analysis data that depend on the grouping method.

- **Microarray(s): Clone Number, Intensity, Paths**
- **Microarray pair(s): Clone Number, Intensity I, Intensity II, Ratios, Differences, Paths**
- **Condition(s): Clone Number, Intensity, Paths**
- **Condition pair(s): Clone Number, Intensity I, Intensity II, Ratios, Differences, Paths**

Microarray or Condition



Microarray Pairs or Condition Pairs



The **clone number** is the index of the clone in the microarray (the microarray address is the filter type plus the clone number, *e. g.*, 'GF200 100'). For GeneFilters® microarrays, the clone number is ordered by field, grid, row, and column.

Intensity is the normalized intensity for a clone. This value represents an average for conditions with repeats. The Intensity variable is labeled I or II for the first or second set in a paired analysis. Paths are discussed in detail below.

The analysis data is pluggable. New data fields that derive from the base types (intensity, ratios, *et cetera*) can be added to the basic set by adding a plug-in. The outlier plug-in, for example, appears for each paired analysis. An outlier describes clones that yield consistently high or low ratios across multiple pairs of microarrays / conditions. An outlier index near 1.0 indicates consistently high ratios and an outlier index near -1 indicates consistently low ratios. The outlier is calculated by first sorting the ratios for each microarray / condition pair and then scaling the sort index from -1 to 1, corresponding to the minimum-through-maximum ratio in the current pair (the unscaled sort index would vary from 1 to the number of clones). The corresponding scaled sort indices for each clone are averaged across the pairs to yield the outlier index.

The **Chen test** is a statistical analysis plug-in that determines whether two sampled intensities are different, based on a desired confidence level. When a clone is not filtered at a confidence level, then the difference in intensity between two samples is statistically significant (at the specified confidence level). Unlike the t-Test, this test is applied to paired data (microarray pair or condition pair grouping, control and experimental data). The Chen test plug-in is extended from a manuscript by Yidong Chen (Chen Y *et al., J. Biomedical Optics* 2(4), 364-374, October 1997, ISBN 1083-3668). This statistical test is based on an assumption that the coefficient of variation is constant across microarray / condition data points. **Before using the plug-in, carefully review this manuscript, including the assumptions involved in derivation of the test**. The manuscript limits the test to an assumed distribution of the data. The Chen test plug-in, as an option, extends the test to a distribution free form.

The **t-Test** is a statistical analysis plug-in that determines whether two sampled intensities are different, based on a desired confidence level. When a clone is not filtered at a confidence level, then the difference in intensity between two samples is statistically significant (at the specified confidence level). This test is applied to paired data with repeats and is limited to condition pairs. The t-Test plug-in is an implementation of the commonly used **Unrelated t-Test** (Student's test). Unlike the Chen test, this test involves a single microarray sampled multiple times. It determines whether the difference between two condition-averaged clones is significant compared to the standard deviation of the sampled intensities for each of the condition-averaged clones. This plug-in offers both a Gaussian distributed and a distribution free form.

Analysis of variance (**ANOVA**) is used to test if the differences in mean intensity values obtained under different experimental conditions are statistically significant. ANOVA compares the variance of the data calculated within conditions to that across conditions. If the variances are not the same, then it is an indication that the means are different. The t-Test is a special case of ANOVA for two conditions. The algorithm here is formulated in way that allows the experimenter to create and install a Java plug-in to exploit experimental design.

ANOVA in Pathways™ has been designed to accommodate different experimental designs through the use of plug-ins. A plug-in is provided for One-Way Analysis. If the data will support an analysis (three or more conditions and two or more samples for at least one clone in each condition) then the ANOVA entry will appear in the data filter window. The filter control sets confidence levels of 99.9%, 99%, 95%, 90%, 75%, and 50%, and "any" which shows all points. At a given confidence level, the filter will remove from the display those spots whose value of the test statistic is less than the critical value for the selected confidence.

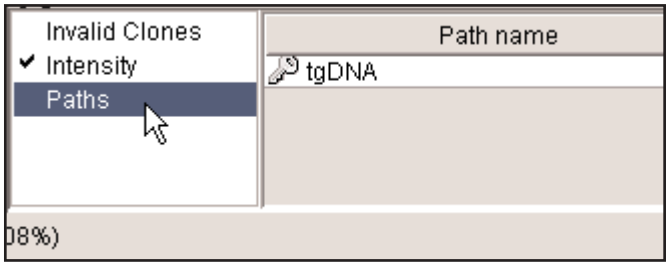
9.2 Data Filtering

Microarray experiments generate massive data sets. A single microarray can hold 5,000 or more clones and even the simplest experiments uses two or more microarrays. In general, these large data sets must be reduced before the underlying significant data sets become apparent.

Data filtering reduces the data set based on a specified set of criteria to generate a more manageable set of data. Data filtering can take various forms.

- **Simple data filters establish a threshold for the data. An example of a simple data filter is a requirement that the ratio of normalized intensity be greater than 2.0 or less than 0.5 ('thresholding' a level of up and down regulation).**
- **Statistical data filters reduce the data set by eliminating clones that lie outside a specified significance level.**
- **Path data filters reduce the data set by requiring that clones be either members or non-members of a specified list of clones (a path). Each filter type is described in detail in this chapter.**
- **Invalid clone filters reduce the data set by allowing the researcher to show all clones, only valid clones, or only invalid clones.**

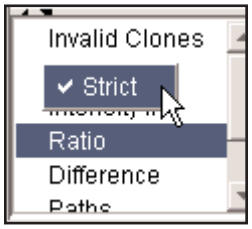
The data displayed in the analysis window are those that have not been filtered out; they are data that meet the criteria. The default behavior for all filters is to not filter any data until the user interacts with the filter (by, for example, adjusting a histogram). A check is next to each active filter in the current analysis window. When the cursor is inside the filter selection window, the current status of the filtered data is displayed as a status message.



9.3 Strict Setting

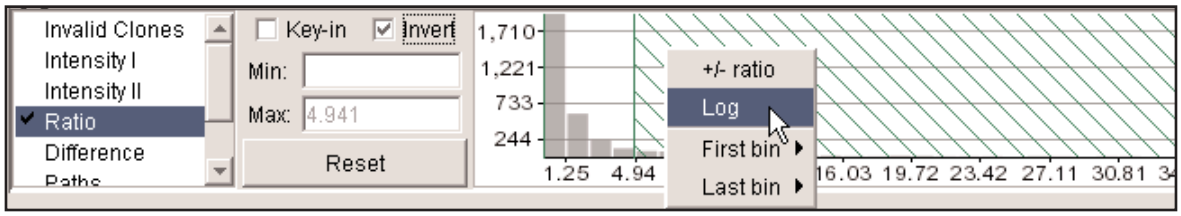
Two data filtering options are available for the case when multiple data sets (multiple microarrays, microarray pairs, conditions or condition pairs) are selected for an analysis window. **Strict data filtering** eliminates a clone from the analysis if the corresponding clone in **any** of the multiple data sets falls outside of the range of the filter. **Non-strict data filtering** eliminates a clone from the analysis only if the corresponding clone in **all** of the multiple data sets falls outside the range of the filter. As an example, consider an analysis window where two microarrays are displayed. If a clone has a normalized intensity of 1 in the first microarray and 2 in the second microarray, a data filter specifying a minimum intensity of 1.5 with the strict setting on filters the clone, while a non-strict setting does not filter the clone.

Strict data filtering is the default. To change the data filtering type, right click on the filter list and select (deselect) the strict option.



9.4 Simple Data Filters

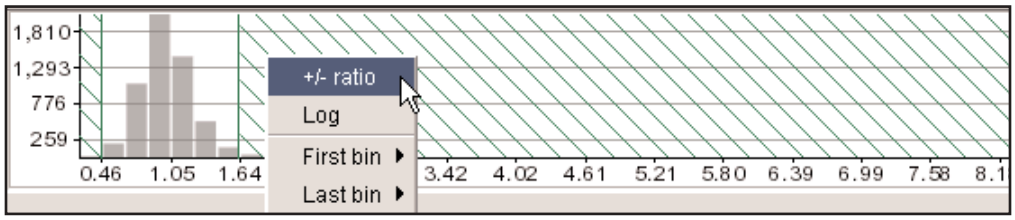
Simple data filters use histograms and min / max value key-ins to limit a variable to a specified range of values. With variables like normalized intensity and intensity ratios, simple data filters reduce the data sets by eliminating data that the user regards as uninteresting.



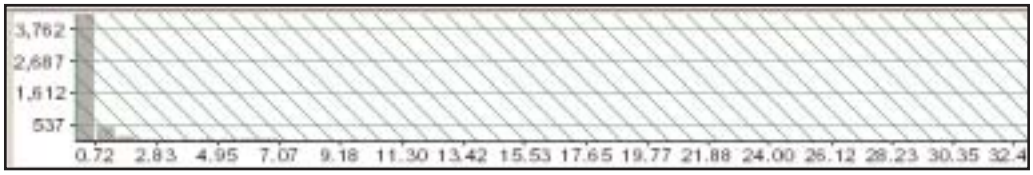
The window above shows a simple data filter applied to the Ratio of normalized intensity. The histogram is being used to filter data from the current analysis window. To use the histogram to filter data, drag the edges of the histogram until the hashed data regions encompass only the desired range of data. To key in limits, check the **Key-in** box and type the min / max limits in the text fields.

The standard mode for the histogram is to include only data between the min / max values specified (Min less than or equal to Data less than or equal to Max). The **Invert** check box selects only data that are outside the min / max bounds (Data less than or equal to Min **or** Data less than or equal to Max). This option affects both the visual histogram filter and the Key-in of min or max values.

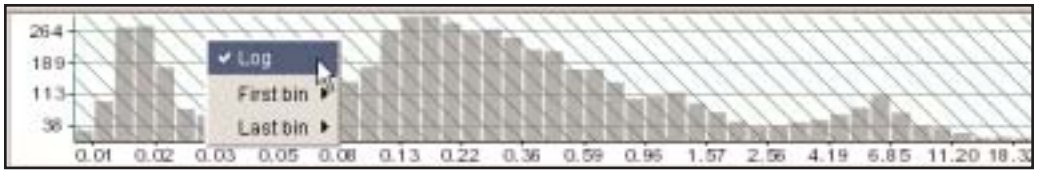
To generate a menu with options for enhanced viewing, right click on the histogram. The **+ / - Ratio** menu item appears for only the Ratio filter. This option reformats the histogram by the upregulation or downregulation format for ratios (if intensity B > intensity A, ratio = B / A; otherwise ratio = -A / B).



The Log option creates bins based on a logarithmic, rather than a linear scheme. This option works well with leveling data in a histogram that has a few large bins and multiple small or empty bins due to one or two data points at the extreme end of the data scale. Another fix for a skewed histogram is to lump a certain amount of the data into the first or last bin, and use a linear scale for the rest of the histogram.



Linear Bins



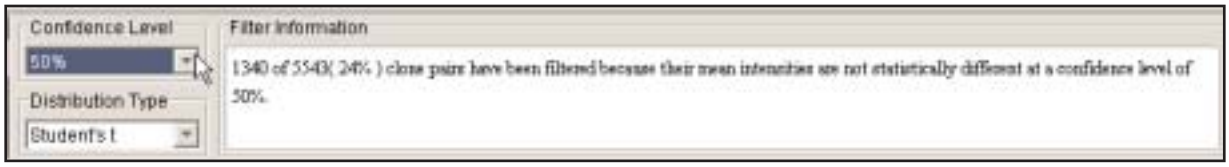
Logarithmic Bins

10 % of Data in Last Bin

9.5 Statistical Data Filters

Statistical data filters reduce the data set by eliminating data falling outside a specified statistical confidence level. The unrelated (Student's) **t-test** analyzes each clone in a condition pair to determine whether the difference in intensities is statistically significant (this test requires at least two measurements per clone). Likewise, the **Chen test** determines whether the difference in intensity of each clone in a pair is statistically significant, but it does not require repeated measurements. Finally, the analysis of variance (**ANOVA**) test determines whether the means of three samples differ significantly from one another.

Each statistical test may have more than one distribution type, as shown below.


Confidence Level
50%
Distribution Type
Student's t
Filter Information
1340 of 5543(24%) clone pairs have been filtered because their mean intensities are not statistically different at a confidence level of 50%.

9.6 Paths

The three **Path types** offer the ability to include or exclude clones from an analysis window.

Microarray address paths identify a clone number on a microarray type. For example, a Microarray address path might specify clones 30, 45, 1000, and 2010 on GF200. This path type is specific; no members of this path are on a GF211 filter, for example. Create microarray address paths manually, through the path editor, or automatically, based on analysis window clones.

Microarray address paths routinely exclude certain clones from analysis. For example, if the thumbnail image of a clone shows an experimental or sampling error, a microarray address path could be created with the address of this clone, and then the clone could be filtered using a path filter.

Clone key paths are similar to microarray address paths, except the clones in this path are listed by the Clone Key (the accession number, when it is available) or by a unique identifier (such as 'tgDNA'). Clone key paths are not microarray specific, and they are therefore a preferred means of identifying clones. Create clone key paths manually through the path editor or automatically based on the clones in an analysis window.

Clone key paths identify a set of clones for further research. For example, if an experiment shows that a set of clones is consistently upregulated, then these clones could be isolated into a clone key path for investigation on further experiments. Likewise, if a researcher has a set of clones on which their efforts are focused, then a clone key path would be created with keys for this set of clones.

Automated search paths create a dynamic path based on keyword searches. Specify the following information.

- **a rule**
- **a field to search**
- **the keyword for which to search**
- **location in the field (start, beginning, in the text)**
- **whether to match or mismatch the keyword**

Multiple rules can be added to a path, and it is possible to select whether to match clones that satisfy the rules or to match clones that satisfy one or more rules.

Automated search paths isolate a research concept. For example, create an automated search path by requesting clones that have the keyword 'cancer' in the clone's description field.
Search paths are dynamic. Whenever a GeneFilters® microarray description is updated (through the Pathways™ update service), automated search paths may change. For example, if Pathways™ data files are updated and new clones have the keyword 'cancer' in their description, they are added to the path automatically.

Paths are matched without regard for the case of the input string (paths are case insensitive).

9.7 Creating a New Path

The Path editor can be accessed from the Quick Start palette, from the Path creation button in an analysis window, or from the Path filter view. When creating a path in the Path editor or in the Path filter window, the **New Path** dialog box appears.

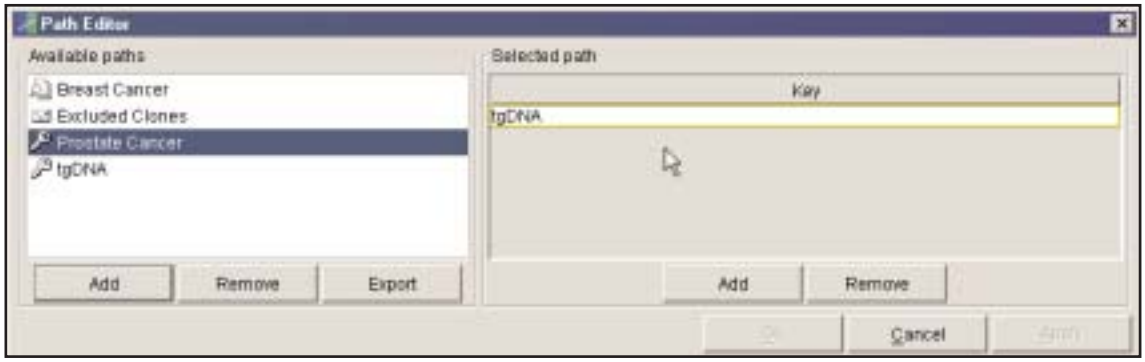
This dialog box allows creation of an empty path, copy an existing path, or import a path from a text file (key and address paths only). Start the new path creation by typing the new path name in the **Name** text field. To create an empty, editable path, select the appropriate type in the **New**

tab of the import dialog box, and click *Ok*. To copy an existing path and then edit that path, select a path from the **Copy** tab, and click *Ok*. To import a path (key or address), select the **Import** tab, select a file and path type, and click *Ok*. To import a key path, create a text file with a single column of data representing a list of clone keys. To import an address path, create a three column text file representing a list of microarray brand, type, and clone numbers (*e. g.*, "GF Description", "GF200", "2" for the second clone in a GF200 microarray). Separate the columns in the file by commas.

To create a new path from any analysis window, click the *New Path* button on the analysis toolbar. The new path contains the clones that are not filtered in the current analysis window. A window appears and prompts the user to enter the name for the new path and whether the path is stored by clone key or by microarray address.

9.8 Editing Paths

Once a new path has been added or an existing path has been selected for edit, the **Path Editor** dialog box appears automatically.

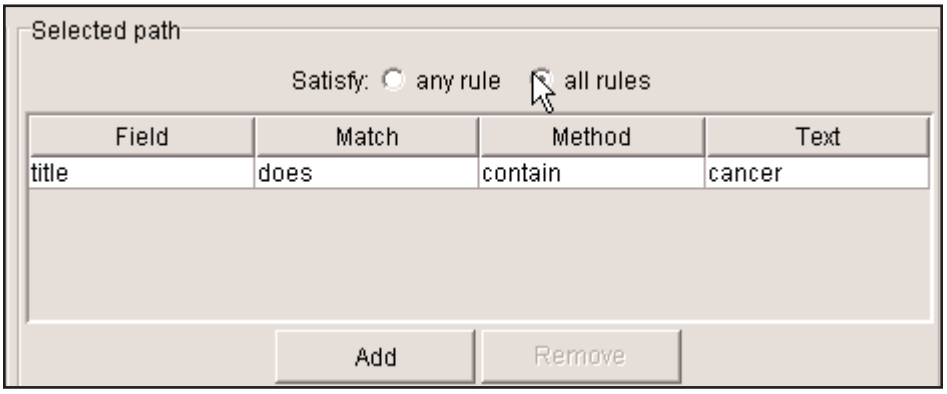


The left portion of the Path editor shows the available paths and includes options for adding new paths, removing paths, and exporting paths to a comma separated text file for use in spreadsheets.

For the Path icons, an envelope is used to depict clone address paths, a key is used to depict clone key paths, and a magnifying glass is used to show automated search paths.

The right portion of the Path Editor is specific to the type of path being edited. Clone key and microarray address paths display a list of keys or a list of entries consisting of (Microarray Brand, Microarray Type, Clone Number), respectively. Entries can be added and removed using the *Add* or *Remove* buttons, respectively.

The Automated Search editor displays a list of rules for the current path along with a radio button to select how these rules are applied.



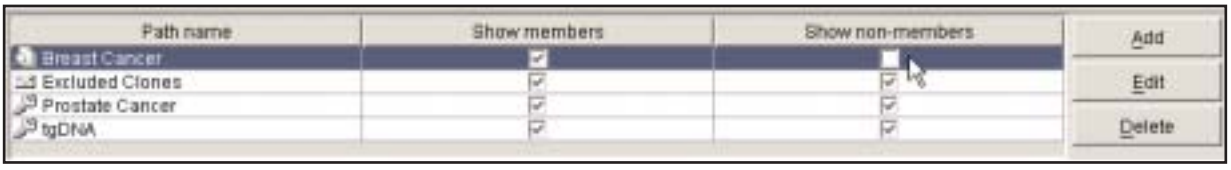
When the path is set to satisfy **any rule,** a clone is included in this path when any of the rules are satisfied. When the path is set to satisfy **all rules**, a clone is included in this path only when all the rules are satisfied. Each rule requires four inputs.

- **Field - the meta data field to use for this rule.**
- **Match - field that specifies whether or not clones must follow this rule**
- **Method - conditions for the text**
  - **as a substring in the field (contain),**
  - **at the beginning of the field (start with),**
  - **as the end of the field (end with), or**
  - **for an exact match (equal)**
- **Text - the text for which to search in the clone data field.**

After editing a Path, click *Ok* to accept any changes and close the dialog box, *Cancel* to revert any changes, or *Apply* to apply any changes to the current analysis, but leave the dialog box open.

9.9 Path Filtering

Path filtering allows including or excluding members from paths. The Path filter window offers a list of paths and check boxes for showing members or non-members of the Path.



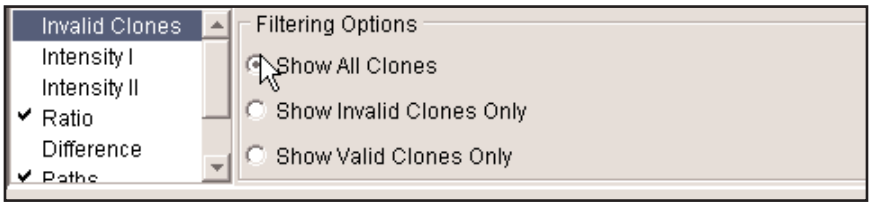
The default behavior is to show both members and non-members for all Paths. To include only members of a path, uncheck the **Show non-members** check box. To include only non-members of a path, uncheck the **Show members** check box. Unchecking both boxes for a path excludes all data points, because all points can be categorized as being either members or non-members of a path.

When path filtering is applied to more than one path, the results set includes the intersection of the modified filters. For example, showing only members of the path "Breast" and non-members of the path "Cancer" yields clones that are in the Breast path but not the Cancer path. Showing only members of the Breast and Cancer paths would yield clones that are in both the Breast and Cancer paths.

9.10 Invalid Clone Filtering

During microarray analysis, some clones do not provide valid sampled data. Multiple factors, including less than optimal experimental parameters, a damaged microarray, or the subjective opinion of the researcher, may render some clones unusable for the analysis process. Pathways™ provides several tools to flag and keep track of such invalid clones. An individual clone marked as invalid will not be automatically excluded from the analysis process but can be excluded using the invalid clone filter. If an invalid clone is used for the calculation of an average intensity during condition analysis, the resulting combined spot will also be marked as invalid.

Invalid Clone filtering allows including or excluding specific clones from the data set. The Invalid Clone filter window offers three options for showing all clones, showing invalid clones only, or showing valid clones only.



Clones marked as invalid from in a project are seen as such in the current project only. Invalid clones are marked as such with crosses if the **Mark invalid clones** button in the upper right corner of the workspace is selected.

There are two places where the user can mark clones as invalid. If the user marks a clone as invalid during the data analysis process, the clone will be treated as invalid only within the current project. The user may also mark a clone as invalid during the image import process (using the Pathways™ Framework importer). If the user marks a clone as invalid during the image import process, any project using the microarray will display this clone as invalid (refer to Chapter 6 for more information about marking invalid clones during the image import process).

Chapter 10: Reports and Exporting Data

10.1 Pathways™ Reporting

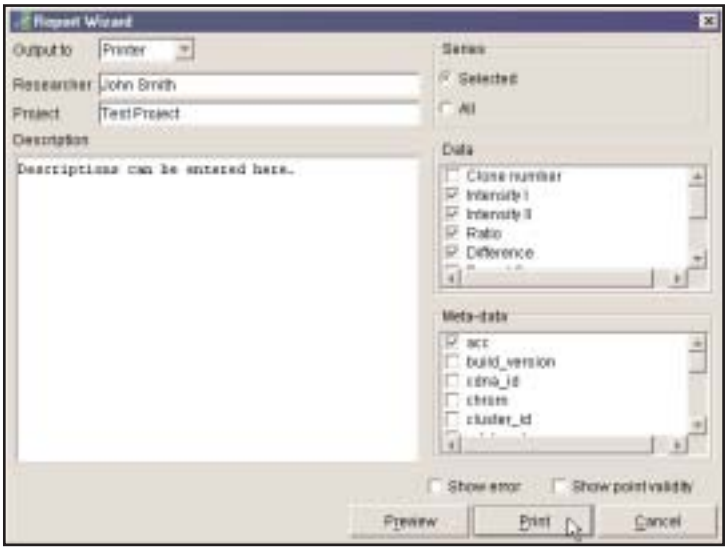
Pathways™ supports reporting / exporting data from all analysis windows. Reports include clones that are not filtered in the current analysis window. The report can contain any combination of data sources (such as intensity, ratios, *et cetera*) and meta data (accession, title, *et cetera*).

There are four options for output.

- **Printer - send report to printer**
- **PDF file - save report in Adobe Portable Document Format (PDF)**
- **HTML file - save report in html format for web viewing**
- **CSV file - save the report as a comma separated value (CSV) for export to spread sheets like Microsoft Excel.**

10.2 Report Wizard

Pathways™ analysis windows support reporting in the toolbar by clicking the *Report* button, , which generates the **Report Wizard** dialog box. For comparison analysis, the Report Wizard dialog box appears.



The output format (printer, PDF file, HTML file, CSV file) can be changed through the **Output to** selection. For the printer output format, a **Preview** option is available. The **Researcher**,

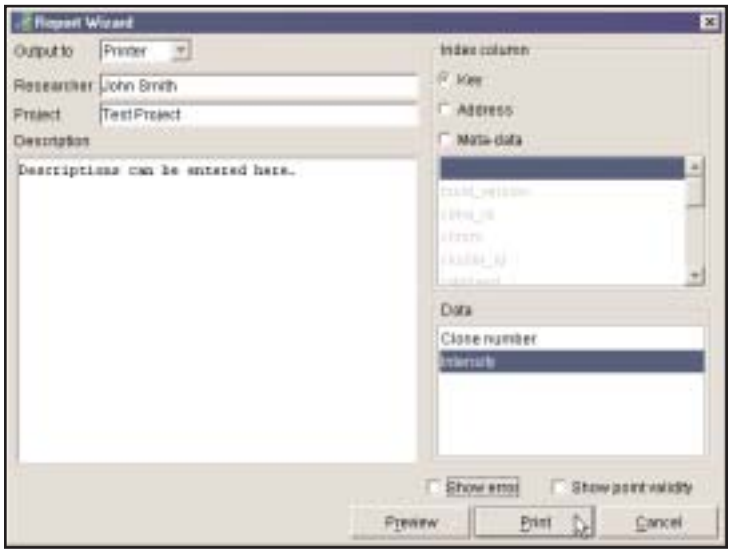
Project, and **Description** fields are editable. The **Series** section contains options for printing the **Selected** experiment or **All** experiments. Selecting **All** shows data for all experiments in a single table and should only be done when outputting to a CSV file. The check boxes on the right of the dialog box allow customization of the report to include the data source (intensity, ratios, *et cetera*) and meta data (accession, title, *et cetera*).

The **Show error** option on the Report Wizard adds a report column for the standard deviation of a condition's intensity. The **Show point validity** option adds a report column for whether or not points are flagged as invalid. If the user wishes to exclude invalid points from the report, apply the invalid clone filter before generating the report.

When the report is configured, click the *Print* button. When the output is being sent to the printer, a Print Preview window appears; from this window, the document can be sent to the printer. When the output is being sent to a file, a file chooser dialog box appears.

The report formatting that generates the printer, PDF, and HTML output is time consuming with large data sets. ResGen™ recommends including no more than 250 clones in the formatted reports. The CSV file does not require any special formatting, and it can be used with an arbitrary number of clones.

The profiling and clustering versions of the Report Wizard dialog box are slightly different from the Comparison Wizard dialog box.



For profiling and clustering, a single 'index' column is selected followed by a data source selection. The index column can be the clone key, microarray address, or any meta data field. A single data column value can be selected for the report. This data column is displayed for each item (microarray, microarray pair, condition, or condition pair) that is included in the analysis. Other functionality is as described for the comparison report dialog box.

Book V: Pathways™ Analysis

Chapter 11: Comparison

11.1 Introduction to Comparison Analysis

Comparison analysis reviews the data in a single condition (or microarray) or compares data between two conditions (or microarrays). A simple comparison analysis would be to display a synthetic image of a single microarray; such an analysis would allow a determination of the peak expression levels in the microarray. A more complex comparison analysis might involve a comparison of one or more pairs of conditions to determine peak upregulation or downregulation between the conditions.

A comparison analysis displays microarray data as synthetic microarrays, scatter plots, or tables. The microarray(s) and condition(s) groupings allow determination of minimum and maximum expression levels or analysis of expression levels of a set of clones (using Paths and other data filters). The microarray pair(s) and condition pair(s) allow determination of the following characteristics.

- **Highly upregulated or downregulated clones**
- **Analysis of ratios**
- **Differences between a set of clones**

11.2 Comparison Toolbar

The comparison toolbar offers functions for comparison windows.

The first three icons in the toolbar enable the **synthetic microarray**, **scatter plot**, and **table** views, respectively (more about these below).

The **path** icon creates a path based on the data remaining after the active filters are applied. A dialog box appears and requests the name for the path and whether the path is stored by microarray address (location in the current microarray type) or clone key (accession or other unique identifier). The path icon is the fourth icon on the toolbar.

The **save image** icon (camera) saves an image of the current data view. Images can be saved in either JPEG or PNG formats. This icon is disabled for the table view. The save image icon is the fifth icon on the toolbar.

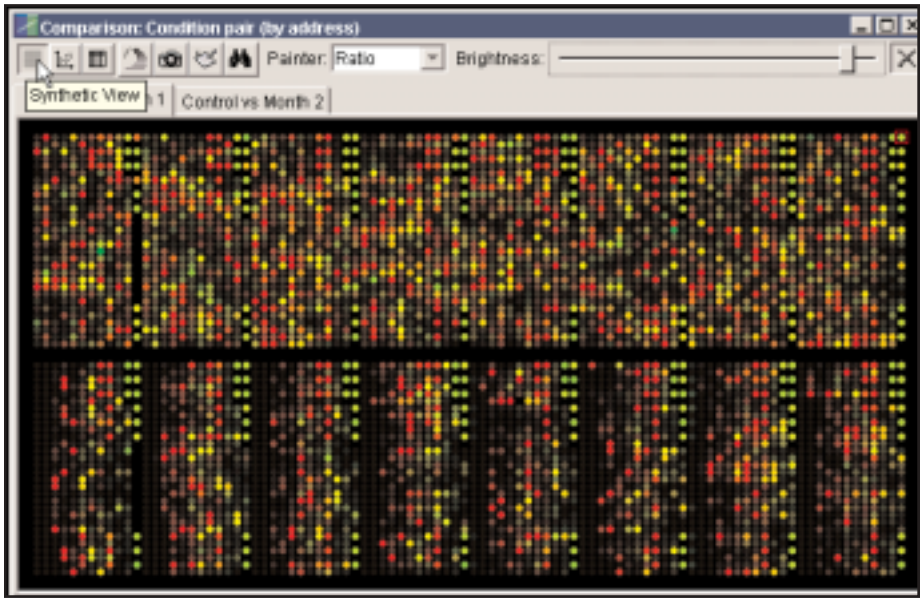
The **report** icon creates reports or export data based on the remaining data after the active filters are applied. The report icon is the sixth icon on the toolbar.

The **find** icon (binoculars) locates clones in the current data view. The find dialog box appears and allows searching for clones by entering a clone key, microarray address, or path. The find icon is the last icon on the toolbar.

Detail views are available in data views by clicking on a data point. In addition, the clone selection can be moved around in the synthetic microarray view using the arrow keys.

11.3 Synthetic Microarray

The first data view for comparison analysis is the **synthetic microarray** view.



A synthetic microarray shows analysis data as colored spots that are arranged in the same pattern as that of the microarray. When multiple microarrays or multiple conditions are presented, the different microarrays appear in tabbed panels as shown in the **Comparison: Condition pair** window.

The synthetic microarray view is not available if the framework being used does not supply geometry (X and Y coordinates).

The coloring on synthetic views depends on **painters** selected from the toolbar.

For microarray(s) or condition(s), the synthetic array shows expression levels. Three painters are available.

1 **Color: highest normalized intensity is bright red, lowest is dim blue**

2 **White on black: highest normalized intensity is white, lowest is black**

3 **Black on white: highest normalized intensity is black, lowest is white.**

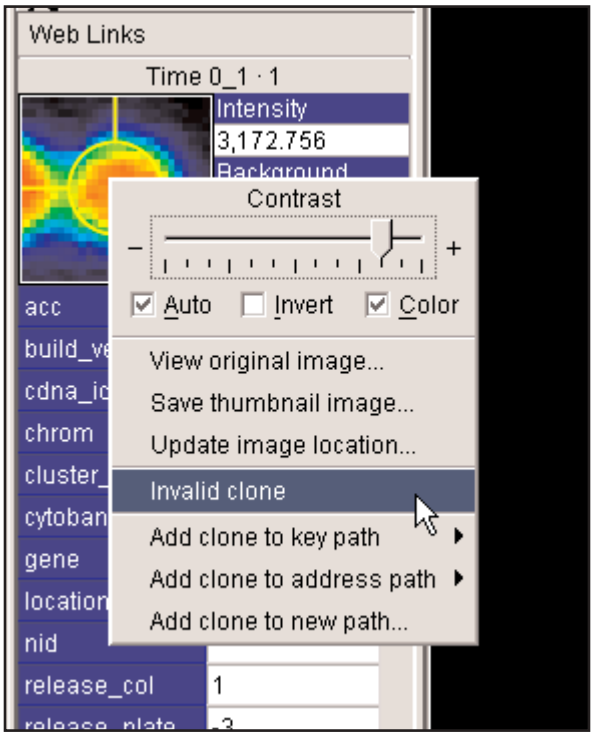
For **microarray pair(s) or condition pair(s), the synthetic array shows upregulation or downregulation**. Two painters are available.

1 **Ratio: upregulation (high ratio) is green, downregulation (low ratio) is red, brightness is relative to the normalized intensity of contributing spots**

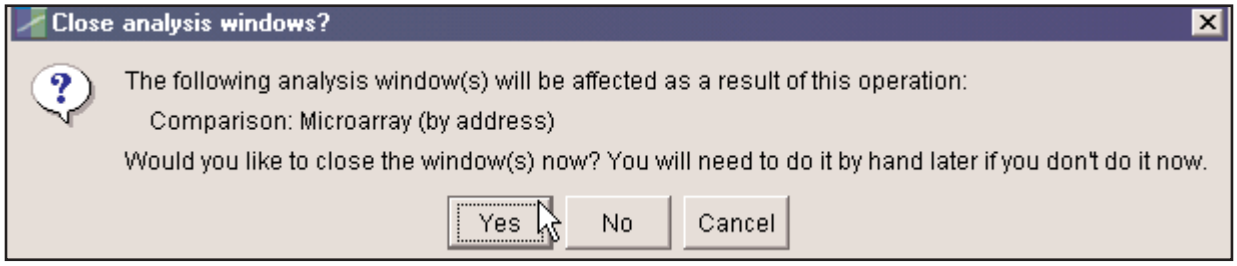
2 **Difference: upregulation (high difference) is red, downregulation (low difference) is blue, brightness is relative to the normalized intensity of contributing spots**

A **Brightness** slider increases spot brightness. Moving the slider to the right allows viewing low intensity spots.

The synthetic microarray may also display invalid clones. Once a microarray is added to a project, individual spots can be marked as invalid by right-clicking on the detailed view of a selected clone and selecting the **Invalid clone** option.



If an opened analysis window is affected by such a change, a dialog box appears, and it lists relevant analysis windows and prompts for an action.

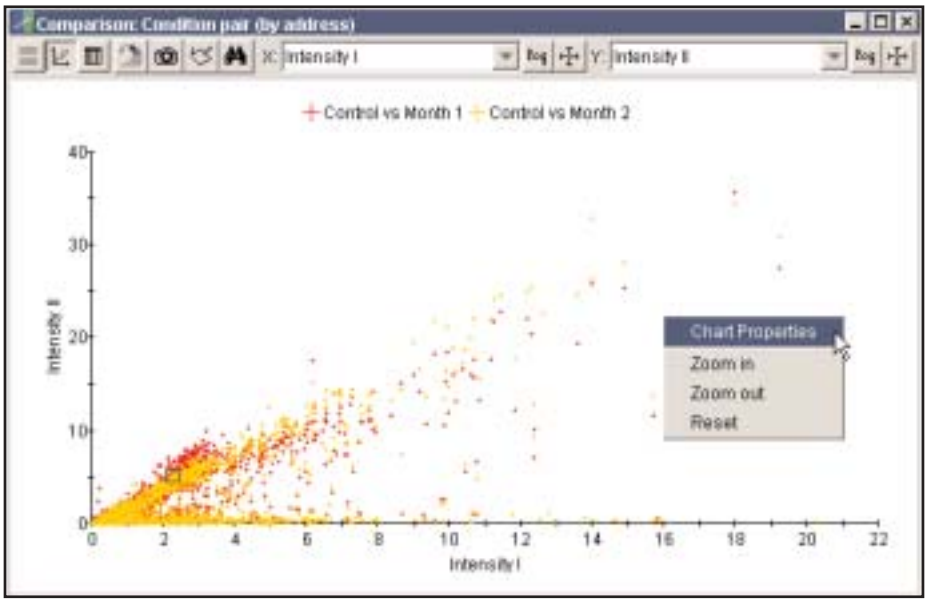

Close analysis windows?
The following analysis window(s) will be affected as a result of this operation:
Comparison: Microarray (by address)
Would you like to close the window(s) now? You will need to do it by hand later if you don't do it now.
Yes
No
Cancel

Select **Yes** to automatically close relevant windows. To mark several other spots as invalid, select **No**. Those windows must be closed and recreated later to take effect.

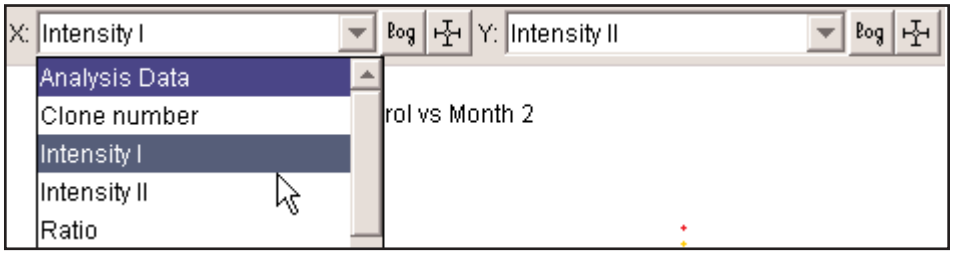
Spots marked as invalid from in a project are seen as such in the current project only.
Invalid clones are marked as such with crosses if the **Mark invalid clones** button in the upper right corner of the view is selected.

11.4 Scatter Plot

The **plot** view enables viewing the data set as a scatter plot with defined axes.



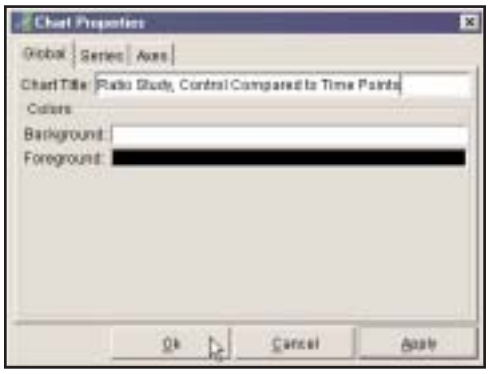
The variable for each axis is selected in the X and Y pulldown menus in the comparison toolbar.



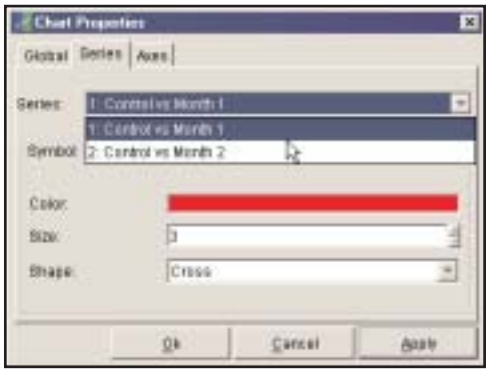
If the current axis variable contains no negative data, then click the **log** button to change the axis to a log scale. When a condition is viewed and the data points have repeats, click the **error bar** button to superimpose error bars on each data point.

11.5 Chart Properties

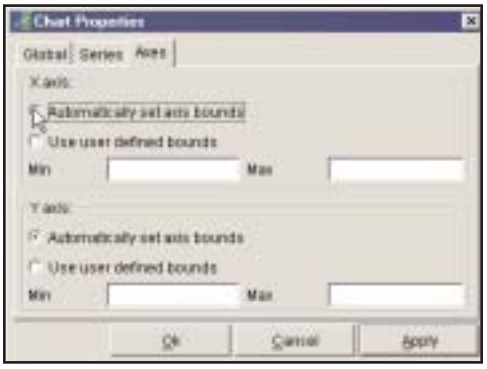
Right clicking on the plot area generates a menu that allows the user to **zoom in / out** on the chart view, to **Reset** the axis limits, and to display the **Chart Properties** dialog box. The Chart Properties dialog box allows customization of the current graph.



The Global tab has options for the chart title and background and foreground colors.



The Series tab allows the symbol color, size, and shape to be specified for each series.



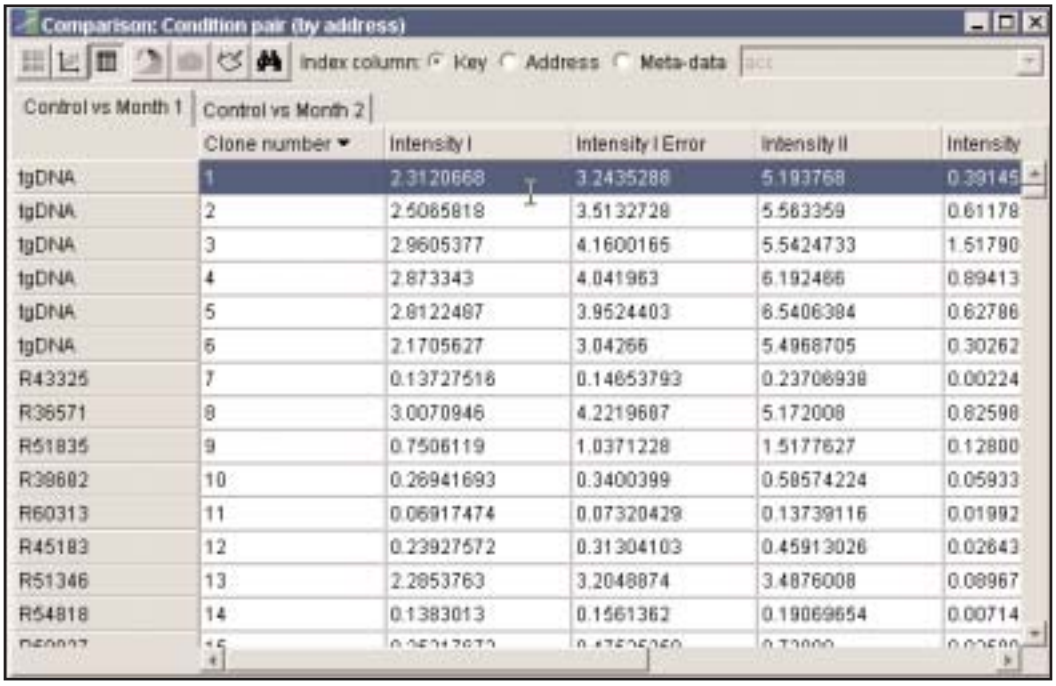
The Axes tab allows the limits for each axis to be specified explicitly; the default option automatically scales the axes.

To apply the modifications, click *Apply*. To exit the dialog box without applying changes, click *Cancel*. To exit the dialog box and apply changes, click *Ok*.

Control + drag pans the chart on the screen. **Shift + drag** creates a region for zooming.

11.6 Table

The table view for comparison analysis presents the microarray data in spreadsheet-like format.



	Clone number	Intensity I	Intensity I Error	Intensity II	Intensity
tgDNA	1	2.3120668	3.2435288	5.193768	0.39145
tgDNA	2	2.5065818	3.5132728	5.583359	0.61178
tgDNA	3	2.9605377	4.1600165	5.5424733	1.51790
tgDNA	4	2.873343	4.041963	6.192466	0.89413
tgDNA	5	2.8122487	3.9524403	6.5406384	0.62786
tgDNA	6	2.1705627	3.04266	5.4968705	0.30262
R43325	7	0.13727516	0.14653793	0.23708938	0.00224
R36571	8	3.0070946	4.2219687	5.172008	0.82598
R51835	9	0.7506119	1.0371228	1.5177627	0.12800
R39602	10	0.26941693	0.3400399	0.58574224	0.05933
R60313	11	0.06917474	0.07320429	0.13739116	0.01992
R45183	12	0.23927572	0.31304103	0.45913026	0.02643
R51346	13	2.2853763	3.2048874	3.4876008	0.08967
R54818	14	0.1383013	0.1561362	0.19069654	0.00714

When multiple microarrays or multiple conditions are presented, microarrays appear in tabbed panels as shown above. Each column can be sorted by clicking the label at the top of the column. Clicking the label again toggles between descending and ascending ordering.

The **index column** (labels on left side of the table) can be modified using the buttons at the top of the table. Options for indexing include the clone key, microarray address, or a meta data item.

Chapter 12: Profiling

12.1 Introduction to Profiling Analysis

Profiling analysis determines the data trends from one experiment to the next. One example of profiling analysis is plotting the intensity of genes associated with cancer at multiple times in a study.

The “microarray(s)” or “condition(s)” groupings allow determination of trends in the expression levels of the clones from one experiment to the next. The “microarray pair(s)” or “condition pair(s)” groupings allow determination of trends in the upregulation or downregulation of the clones between pairs of experiments.

12.2 Profiling Toolbar

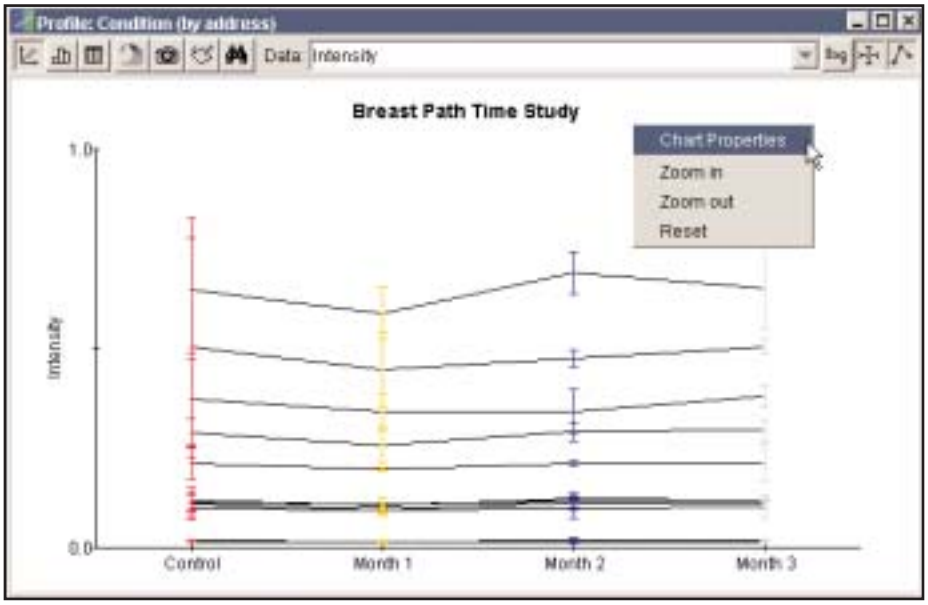
The profiling toolbar is present for profiling views.

The first three buttons represent the **plot**, **bar chart**, and **table** views for profiling analysis (these buttons are discussed in more detail below).

The remaining four toolbar buttons are the **path**, **save image, report**, and the **find** buttons. Refer to Chapter 11 for details.

12.3 Plot

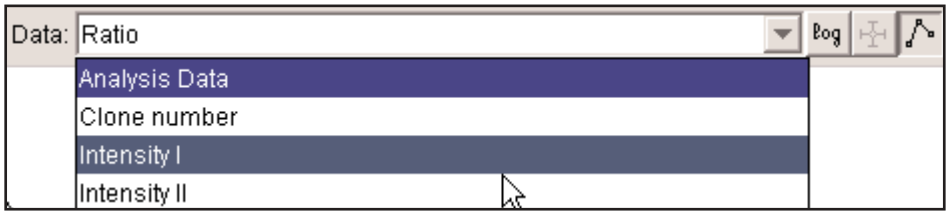
The plot window displays a variable (intensity, ratio, difference, *et cetera*) on the Y axis, while the X axis displays labels for the microarray, microarray pair, condition, or condition pair, depending on the data grouping type.



The foregoing plot used a path filter to isolate only certain clones. Path filtration is advisable when using the plot view, because the plot becomes overcrowded with large data sets.

Clicking the **left** and **right arrow keys** moves a selected clone to the left or right data point for the current clone. For example, if the selected clone is in the '1 Month' condition above, then the left arrow moves the selection to the same clone in the 'Control' condition, and the right arrow moves the selection to the same clone in the '3 Months' condition.

The y axis variable is selected in the **Data** menu in the profiling toolbar.



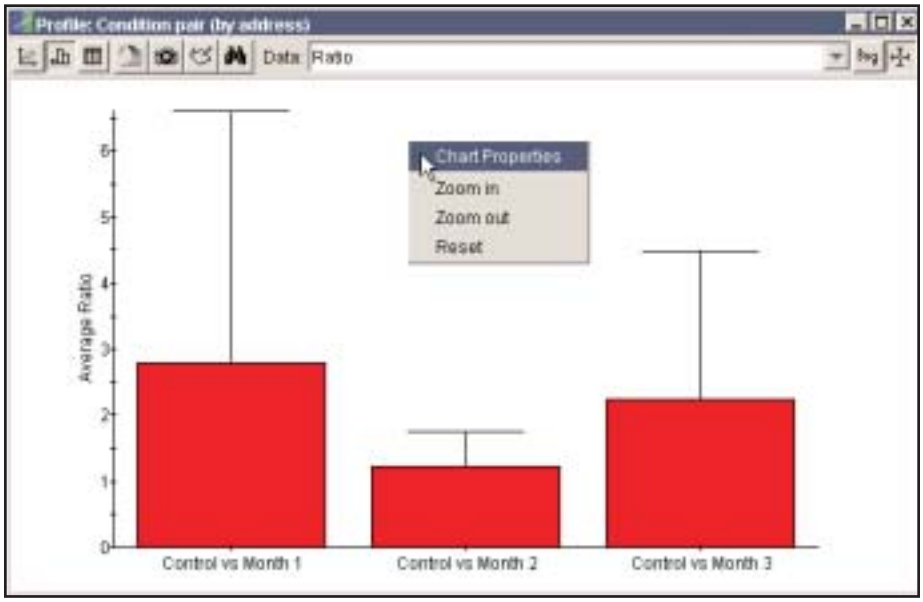
Path data is unavailable for plotting in profile analysis, because there is little meaning in plotting two values on the y axes (Member / Non-Member) *versus* a handful of profile state points.

If the current axis variable does not contain negative data, then the axis can be changed to a log scale using the **log** button. When a condition is being viewed and the data points have repeats, then error bars can be superimposed on each data point using the **error bar** button. Clicking the **connect lines** button toggles between symbols with lines connecting the data points and symbols alone.

Right click on the plot to display the chart menu: **Chart Properties**, **Zoom In**, **Zoom Out**, **Reset** (see Chapter 11 for a detailed explanation of the Chart Properties dialog). **Control + drag** pans the chart on the screen. **Shift + drag** creates a region for zooming.

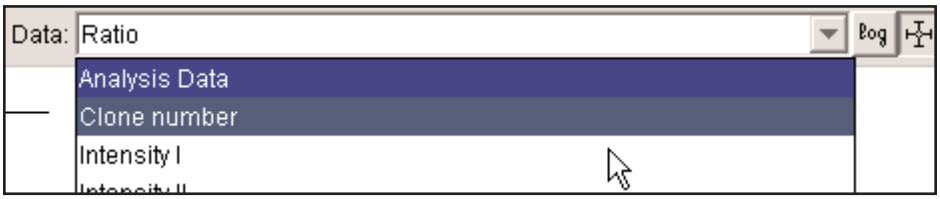
12.4 Bar Charts

Bar charts provide the summary of a variable at each state in the profile analysis.



The height of each bar represents the average value of a variable **for clones that have not been removed by data filters**.

The y axis variable is selected in the **Data** pulldown menus in the profiling toolbar.



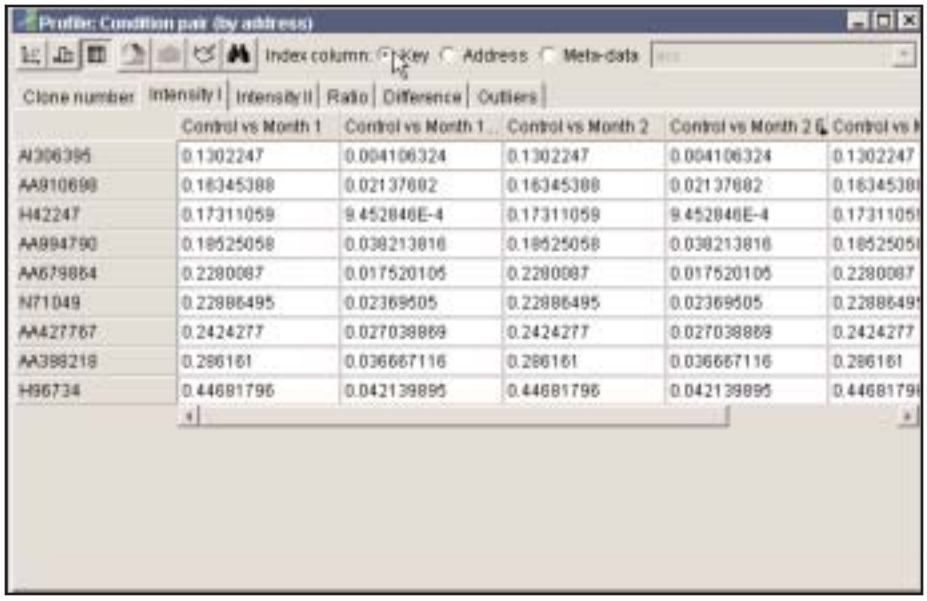
If the current axis variable contains no negative data, then the axis can be changed to a log scale using the **log** button. The **error bar** button displays the error bars representing the standard

deviation of the data points. These deviations represent each state's average value. This result is not the same as error bars for repeated clones that are displayed in other analysis windows.

Right clicking on the bar chart displays the standard chart controls, including the Chart Properties dialog box. This version of the Chart Properties dialog box is slightly different from the version for condition comparison. The bar color has been added to the Global tab in the Chart Properties dialog box, and the Series tab is not present, because no additional specifications are necessary for each series.

12.5 Table

The table view presents the profiled data in a spreadsheet format.



Items of analysis data (*e.g.* intensity) are displayed in a tab panel as shown above. Each column can be sorted by clicking the label at the top of the column. Clicking the label again toggles between descending and ascending sort ordering.

The **index column** (labels on left side of the table) can be modified using the radio buttons at the top of the table. Options for indexing include the clone key, microarray address, or any meta data item.

Chapter 13: Clustering

13.1 Introduction to Clustering

Clustering analysis automatically generates associations between clones in an analysis set. For example, clustering could find clones that respond similarly (upregulation or downregulation) over the course of a study. Mathematically, clustering algorithms locate points that are close together in a multi-dimensional clustering data space.

Clustering is complex, and a complete explanation of the clustering process is beyond the scope of this guide. The following sections are an introduction and overview of clustering analysis.

Clustering data space is based on the analysis grouping and a variable. For example, a cluster analysis of normalized intensity of three microarrays might group clones that are close together in a three dimensional space defined for each clone as: {intensity in first microarray, intensity in second microarray, intensity in third microarray}. An effective clustering algorithm groups clones that are {bright, bright, bright} across the three microarrays. Likewise, clones appearing as {dark, bright, dark}, {dark, dark, dark}, {bright, bright, dark}, *et cetera* over the three microarrays are grouped together. Therefore, this analysis seeks clones that have similar expression profiles across the three microarrays.

As another example, a time study could be clustered based on ratios between each time point and the control, which is at time zero. Assuming four time points plus the control, the cluster space would be four dimensional: {time 1 / control, time 2 / control, time 3 / control, time 4 / control}. Therefore, the clustering algorithms would group together clones that have a similar upregulation and / or downregulation pattern over the course of the study.

13.2 Clustering Algorithms

Clustering algorithms differ in how they group points together in data space. For example, the **KMeans** algorithm groups data into a specified number of clusters by finding the center of the cluster and assigning data to the nearest cluster center in an iterative fashion. KMeans is a partitional clustering algorithm, because the clustering process consists of finding the best cluster (partition) for each clone.

Hierarchical clustering algorithms work by finding the two closest clones and calling this a cluster. The cluster is assigned a position in the data space that is based on the linkage method. Next, the closest two entities (clone or cluster) are found and grouped into the second cluster. This process repeats until all clones are grouped into clusters.

The self-organizing map (**SOM**) clustering algorithm finds clusters in an input data set by mapping the data onto a two-dimensional array of nodes. Each node contains a reference vector that records the value associated with that node. The data points represent successive expression levels of a clone. The map is constructed by comparing each point in succession with the refer-

ence vector of each node. The node with the reference vector that is nearest an input vector is updated with a weighted combination of the reference and input vectors. This process is repeated over many iterations. The number of iterations in each stage and the initial values are user-defined.

There are many ways to describe the **Distance** between points in data space. The traditional distance calculation is a Euclidian calculation. The squared Euclidian distance places a heavier weighting on distances than the standard Euclidian calculation. The correlation metric is Pearson's correlation coefficient proposed by Eisen *et al*. (1998), and it is analogous to the vector inner product.

13.3 Cluster Visualization

Visualization of clustering is a challenging task. Clusters can be viewed in one of two 'spaces': data space or cluster space. **Data space**, as described above, is defined by the variable and the grouping (*e*. *g*., five conditions = five dimensional space). **Cluster space** is the distance from each clone to a cluster. This representation of the data helps to display how close each clone is to an arbitrary cluster. The number of dimensions is equal to the number of clusters.

Both data and cluster space tend to be greater than three-dimensional space, and they are impossible to view in a traditional sense. Therefore, visualization techniques focus on displaying as much information as possible within the constraints of the two-dimensional computer screen.

13.4 Clustering in Pathways™

In recognition of the complexity of the entire clustering process, Pathways™ clustering analysis has been designed to be highly modular through the use of plug-ins. Plug-ins are available for the following major components.

- **Cluster plug-in: core clustering algorithms, such as KMeans and Hierarchical.**
- **Cluster Distance plug-in: calculates the distance between two clones or between a cluster and a clone, based on the locations in data space (used in tandem with Cluster plug-in).**
- **Cluster Visualization plug-in: takes a cluster calculated by any method and displays the cluster. The plug-in establishes the format of the display (graph or table).**

Therefore, the process of clustering consists of the following components.

1. **Selecting the desired clustering plug-in and specifying any auxiliary information needed**
2. **Selecting the desired visualization technique**

Implemented plug-ins are described in more detail below.

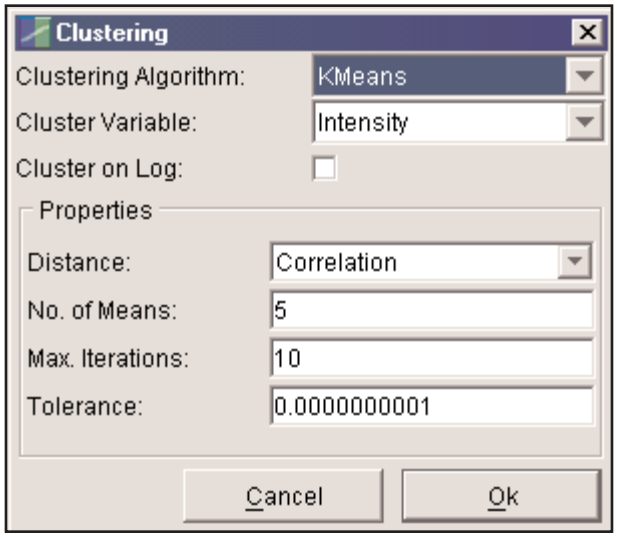
A **cluster filter** is added to the filter view after performing a clustering operation.

The cluster filter has the following four settings.

- **Show all clusters: no filtering**
- **Show clusters in specified range: isolates one or more clusters with a series of specified cluster numbers, *e. g.*, 1 to 5, 10, 25.**
- **Sweep through clusters: a slider bar reviews multiple clusters. As the slider is moved from the left to the right, the first through the last cluster is shown in the cluster visualization window. If the number of visible clusters is set to greater than one, the slider shows multiple clusters around the currently visible cluster.**
- **Isolate cluster for selected clone: shows only that cluster associated with the selected clone. This feature is powerful when it is combined with the Find Clones dialog box. For example, turn on the isolate cluster feature, and use Find Clones to find each clone in a cancer path; the result identifies the clones clustering with each cancer clone.**

13.5 KMeans Clustering

Selecting an analysis grouping from the **Cluster** menu generates a dialog box. The dialog box includes the current clustering algorithms in a pulldown menu.



The **Clustering Algorithm** is the first option in the Clustering dialog box. Available selections are KMeans, Hierarchical, and SOM. The next selection is the **Cluster Variable**. Variables like intensity, ratio, and differences are available, depending on the selected analysis grouping.

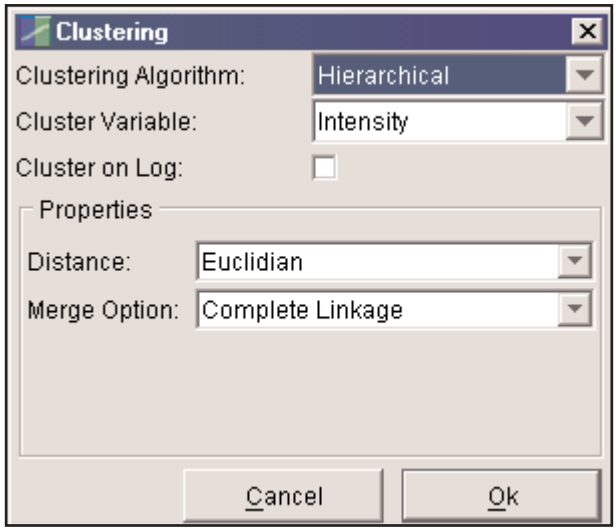
The **Properties** section of the dialog box allows entry of information that is specific for the selected clustering algorithm. For KMeans, specify the following options.

- **Distance: plug-in calculates the cluster distance**
- **Number of means: the number of means (clusters) that the KMeans algorithm generates**
- **Maximum iterations: the KMeans algorithm groups and regroups clones into clusters according to the distance from the clone to the cluster center. The maximum iterations field sets a limit as to the number of iterations on the grouping process.**
- **Tolerance: the iteration process stops if the difference between the 'cost' (average clone to cluster distance) at the current iteration and the cost at the previous iteration is less than this number.**

When the properties are set, click *Ok* to proceed with the cluster analysis.

13.6 Hierarchical Clustering

Hierarchical clustering is a second option for clustering analysis.



As with all clustering algorithms, the cluster variable must be chosen before proceeding with cluster analysis.

In addition to a cluster variable, hierarchical clustering requires the following information.

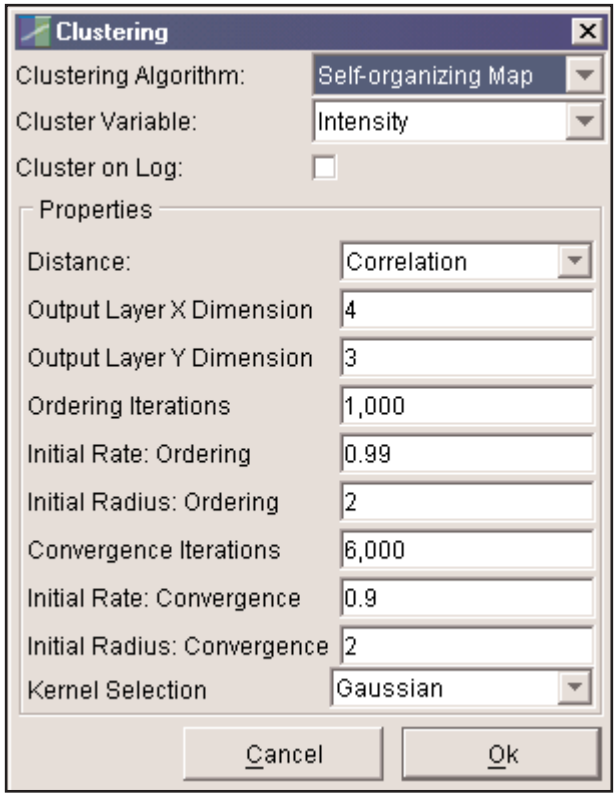
- **Distance: plug-in calculates the cluster distance**
- **Merge Option: linkage method (see below)**

The merge option (linkage) dictates where a new cluster (created when two clones and / or clusters are combined) is located relative to existing clones / clusters. **Single linkage** dictates that the distance between a new cluster and an existing entity is the minimum of the distances between the new cluster's components and the entity in question. **Complete linkage** dictates that the distance between a new cluster and an existing entity is the maximum of the distances between the new cluster's components and the entity in question. **Unweighted** and **weighted averages** calculate the new distance as the average of the previous distances ("weighted averages" weights this average by the number of subentities in a cluster). **Unweighted** and **weighted centroids** use the average of the cluster location in data space to calculate the new distance ("weighted centroids" weights the average by the number of subentities in a cluster).

When the properties are set, click *Ok* to proceed with the cluster analysis.

13.7 SOM Clustering

SOM Clustering is a third option for clustering analysis.



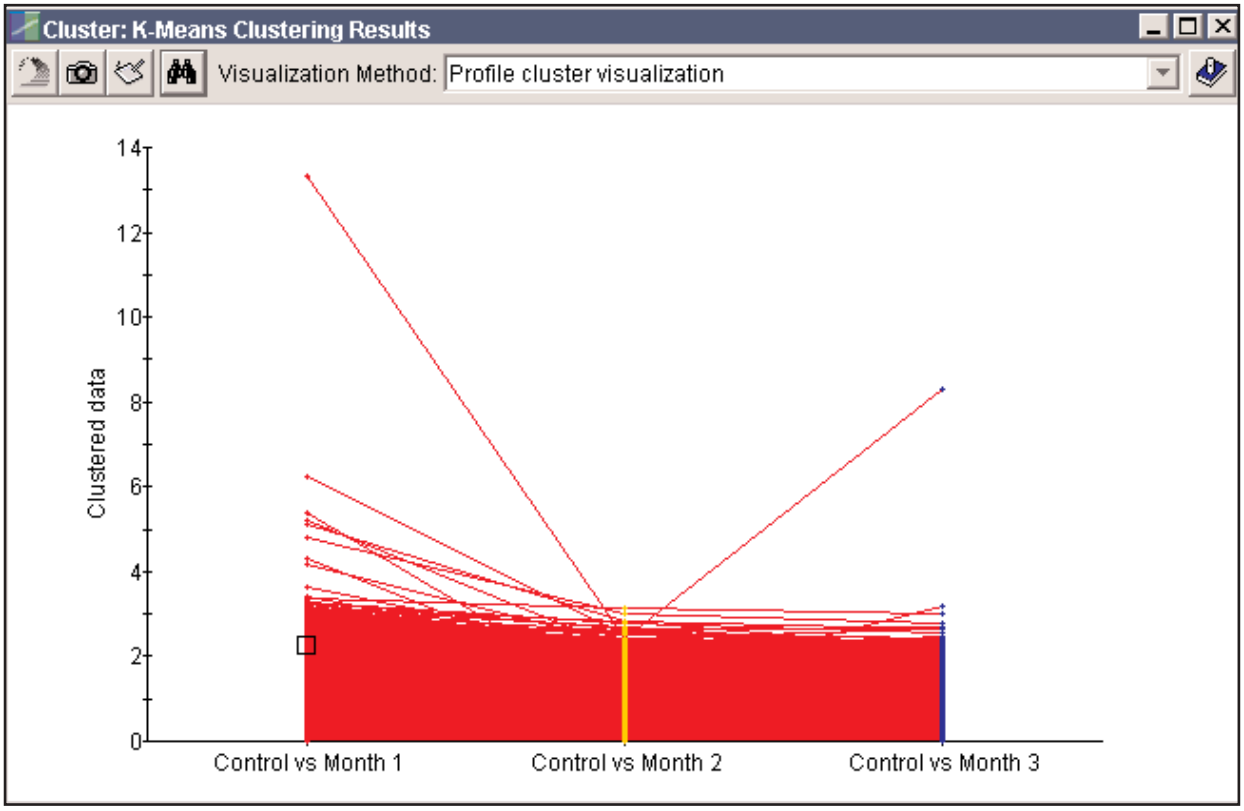
After the cluster variable has been selected, SOM clustering requires the following information.

- **Distance: plug-in calculates the cluster distance**
- **Output Layer X Dimension: number of dimensions for nodes on the X-axis**
- **Output Layer Y Dimension: number of dimensions for nodes on the Y-axis**
- **Ordering Iterations: number of iterations during the ordering phase**
- **Initial Rate: Ordering: initial rate of calculation during the ordering phase. The initial rate decreases monotonically over the duration of the phase to small values near zero.**
- **Initial Radius: Ordering: initial radius of calculation during the ordering phase. The initial radius decreases monotonically over the duration of the phase to small values near zero.**
- **Convergence Iterations: number of iterations during the convergence phase**
- **Initial Rate: Convergence: initial rate of calculation during the convergence phase. The initial rate decreases monotonically over the duration of the phase to small values near zero.**
- **Initial Radius: Convergence: initial radius of calculation during the convergence phase. The initial radius decreases monotonically over the duration of the phase to small values near zero.**
- **Kernel Selection: type of kernel used for calculation. The Cylindrical option weights all nodes equally, while the Gaussian option decreases the weight with increased distance from the updated node.**

When the properties are set, click *Ok* to proceed with the cluster analysis.

13.8 Profile

The profile plug-in displays the cluster data as a connected series of lines, similar to the profile analysis technique.



The profile view shown above has isolated a cluster using a cluster number filter set to show the cluster of a clone associated with cancer.

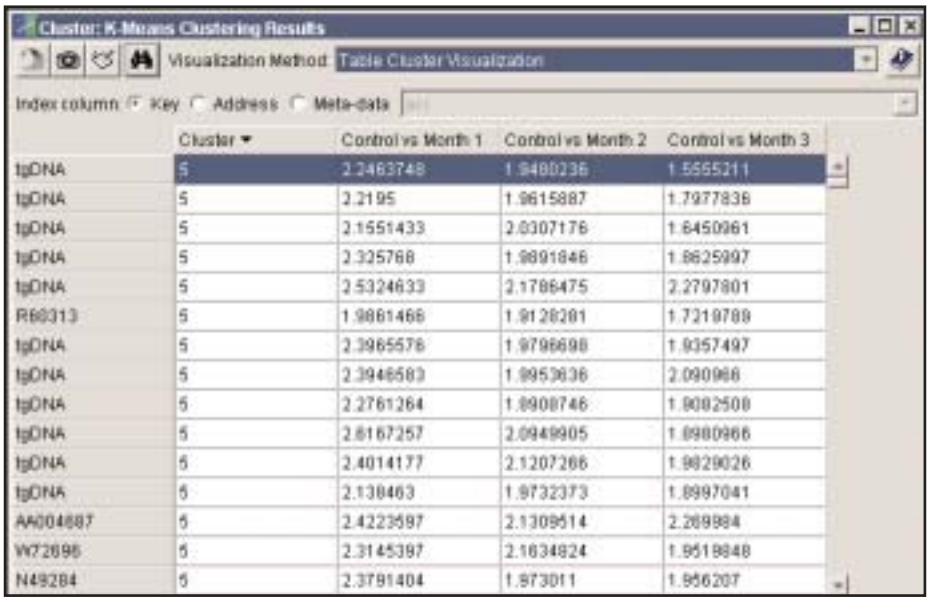
The **Cluster Information** button generates a dialog box with the details of the clustering algorithm that generated the current results. The **Cluster Information** button is available for all cluster visualization techniques.

The **left** and **right arrow keys** moves a selected clone to the left or right data point for the current clone. For example, if the currently selected clone is in the '1 Month' condition above, then the left arrow moves the selection to the same clone in the 'Control' condition, and the right arrow moves the selection to the same clone in the '3 Months' condition.

Right clicking on the plot generates the chart menu: **Chart Properties**, **Zoom In**, **Zoom Out**, **Reset** (see Comparison Analysis for a detailed explanation of the Chart Properties dialog box). **Control + drag** pans the chart. **Shift + drag** creates a region for zooming.

13.9 Tabular

The tabular plug-in displays cluster data in a spreadsheet-like format.



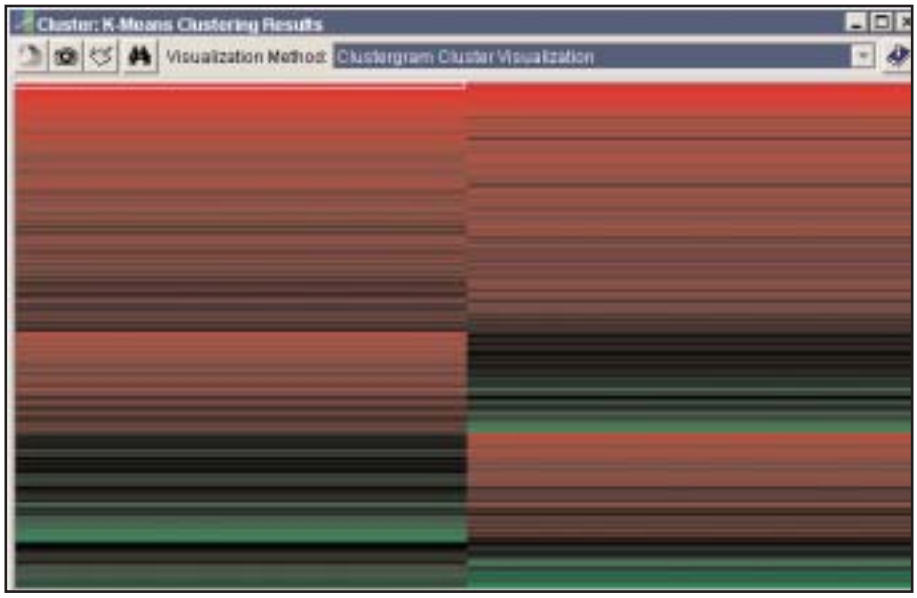
	Cluster	Control vs Month 1	Control vs Month 2	Control vs Month 3
tgDNA	5	2.2463748	1.9480236	1.5555211
tgDNA	5	2.2195	1.9615887	1.7977836
tgDNA	5	2.1551433	2.0307176	1.6450961
tgDNA	5	2.325768	1.9891846	1.8625997
tgDNA	5	2.5324633	2.1786475	2.2797801
R60313	5	1.9861466	1.9128201	1.7219789
tgDNA	5	2.3965576	1.9796698	1.9357497
tgDNA	5	2.3946583	1.9953636	2.090966
tgDNA	5	2.2761264	1.8908746	1.9082508
tgDNA	5	2.6167257	2.0949905	1.8980966
tgDNA	5	2.4014177	2.1207266	1.9829026
tgDNA	5	2.138463	1.9732373	1.8997041
AA004687	5	2.4223597	2.1309514	2.269884
W72696	5	2.3145397	2.1634824	1.9519848
N49284	5	2.3791404	1.973011	1.956207

The rows of the table include the cluster number and data values for contributing microarrays or conditions. Each column can be sorted by clicking the label at the top of the column. Clicking the label again toggles between descending and ascending sort ordering.

The **index column** (labels on left side of the table) can be modified using the buttons at the top of the table. Options include the clone key, microarray address, or a meta data item.

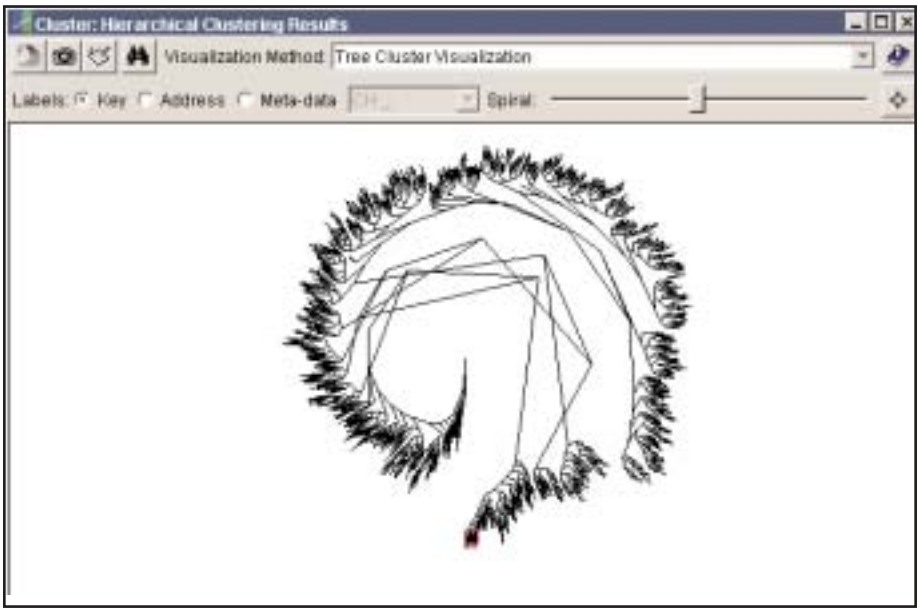
13.10 Clustergram

The **clustergram** plug-in generates the same information as the cluster table above, but colors are used, instead of numbers. The vertical axis of the clustergram represents a clone number and the colors across the clustergram represent a variable for each contributing microarray / condition or microarray / condition pair.



13.11 Hyperbolic tree

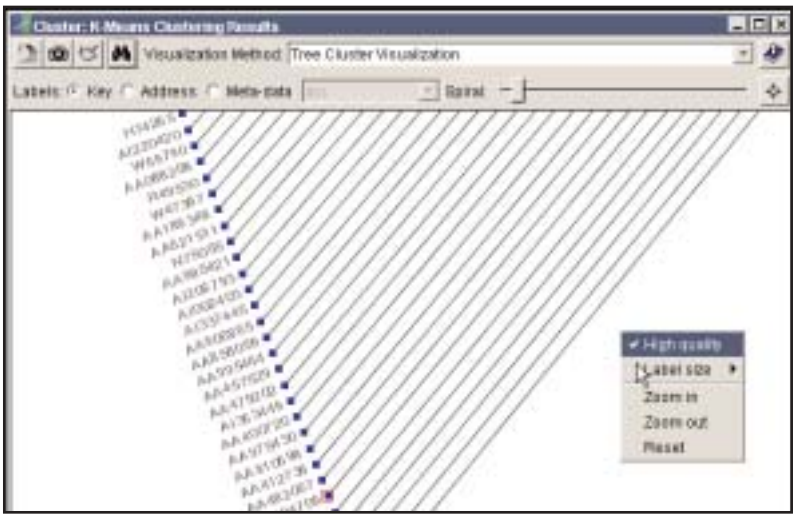
The hyperbolic tree plug-in displays the cluster as a tree of interconnected clones and subclusters.



This tree is mapped onto a hyperbolic surface that displays the tree spiral starting from the root of the cluster tree out to clone nodes. The tightness of the spiral is controlled by a slider on the top bar. Clones can be selected by clicking on the clone or through the **find clones** dialog box. Clicking the **center clone** button centers the current clone in the viewing window. Clones are labeled using the **Clone Key**, **Microarray Address**, or available **Meta data**.

The tree can be magnified by creating a zoom rectangle (**shift + left drag**) or using the up or down arrow keys (zoom in or out). The image can be **panned** using **control + left drag**.

Right clicking generates an options menu for the cluster tree.



This menu allows for **High quality** drawing (slows the drawing process down, but produces higher quality images), **Label size** selection, **Zoom In / Out**, and **Reset** (resets the cluster tree to the original zoom and spiral).

Chapter 14: Pathways™ Data Updates

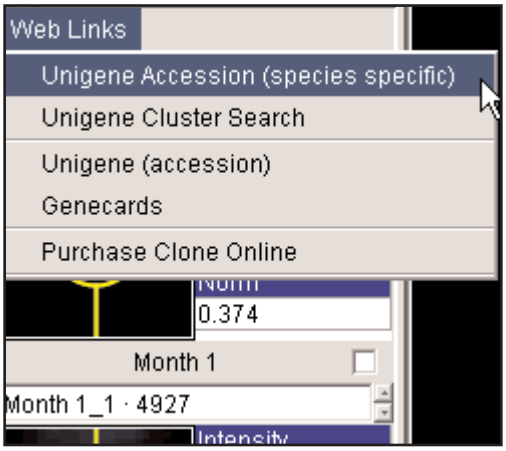
The information on clones in a microarray may change daily as discovery in genetics progresses. Likewise, analysis techniques improve as the field matures. Pathways™ ensures that clone data and analysis techniques are current with the following features.

- **An integrated web browser with links from each clone to critical sites such as Unigene and GenBank®.**
- **Regular updates of GeneFilters® data and Plug-in features through the ResGen™ Pathways™ data server.**

Each of these methods are discussed in detail below.

14.1 Web Links and the Integrated Web Browser

Pathways™ 4 has an integrated web browser that can connect a clone and related web sites.



In addition, a browser window can be accessed through the **Tools→Browser** menu.

The web links that are shown for each clone are implemented through web **plug-ins**. These plug-ins search meta data for each clone to determine whether key fields like the clone accession are present and then dynamically generate web links based on the availability of this data.

14.2 Adding and Editing Web Links

Simple web links are managed using the **Edit Web Links** dialog box (**Tools→Edit Web Links**).

This dialog box allows web links to be added or modified based on a link name and certain key fields. For example, access to Unigene for an accession requires a format like

"http://www.ncbi.nlm.nih.gov/Unigene/query.cgi?TEXT=[acc]",

where '[acc]' corresponds to the acc (accession) field in the meta data for each clone. An item from this table can generate web links. For example, if a fictional link required unigene build information and cluster ID, it would be entered as

"http://someplace.com/mylink?UG=[build_version]&CLUSTER=[cluster_id]".

Determine the appropriate pattern for a web site by visiting the link directly in the Pathways™ browser (or any other web browser) and observing how the URL changes when, for example, different clones are examined online.

To edit an existing link, highlight the link, and click *Edit*. The **Link Name** and **Link URL** fields are activated for editing until *Abort* or *Apply* is clicked (the *Abort* and *Apply* buttons are present during the editing process). To add a new link, click *New*, and enter a link name and URL. When finished, click *Add* or *Abort* to add the new link or abort the edit (the *Add* and *Abort* buttons are present during the editing process).

Certain web links require a specialized plug-in. For example, a Unigene cluster search requires the organism and cluster ID to be separated (the link looks like

"http://....&ORG=organism&CID=clusterid").

The Unigene cluster plug-in splits up the cluster ID (*e. g.*, "Hs.2") to yield the desired web link (*e. g.*, "http://...&ORG=Hs&CID=2").

14.3 Introduction to Pathways™ Updates

Pathways™ data and plug-ins can be updated on a regular basis from the ResGen™ data server. These regular updates offer the following enhancements.

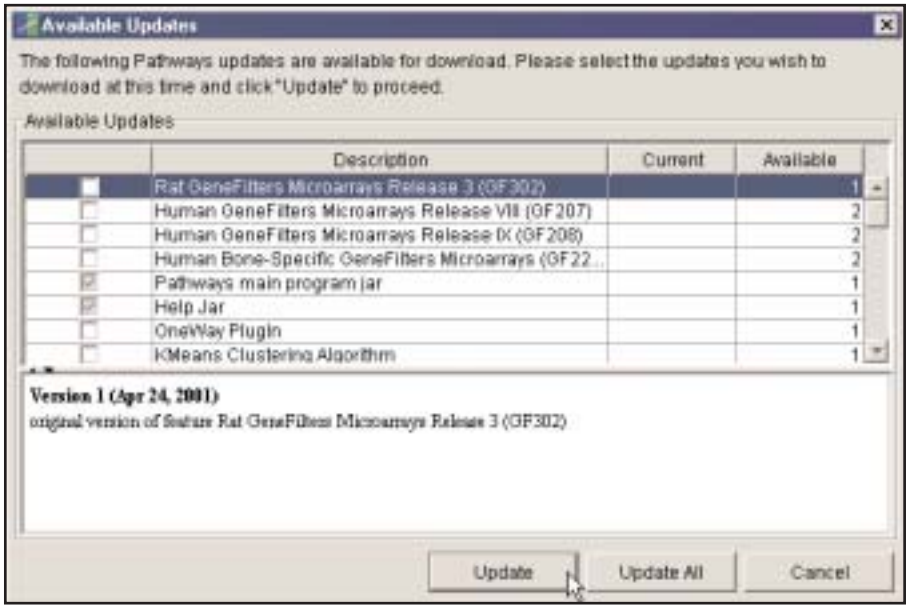
- **Data updates: description files for each GeneFilters® microarray updated to reflect, for example, a new Unigene build.**
- **New microarrays: description files for new GeneFilters® microarray products will be added as they become available.**
- **Plug-In enhancements: enhancements to existing plug-ins are supplied as they become available.**
- **New plug-Ins: as they become available, new plug-ins are added to Pathways™ automatically.**

Data updates are performed by establishing an internet connection between Pathways™ and the ResGen™ data server. Pathways™ sends a message to the data server asking if any updates are available. The data server replies with a list of available updates. Pathways™ retrieves each selected update from the data server. The updated data become available the next time Pathways™ is launched (Pathways™ must be shut down and restarted after the update is complete).

Additional data can be added to the microarray description files; therefore, these files must be treated specially during a Pathways™ update. The description file updates are performed as a merge between the old and new files, so that auxiliary data added to the description files are not lost.

14.4 Launching the Updater

To launch the updater, select **Tools→Update Pathways**. A dialog box appears and indicates that Pathways™ is contacting the ResGen™ data server. Once a successful connection has occurred, Pathways™ displays a list of available updates (Pathways™ data and / or plug-ins).

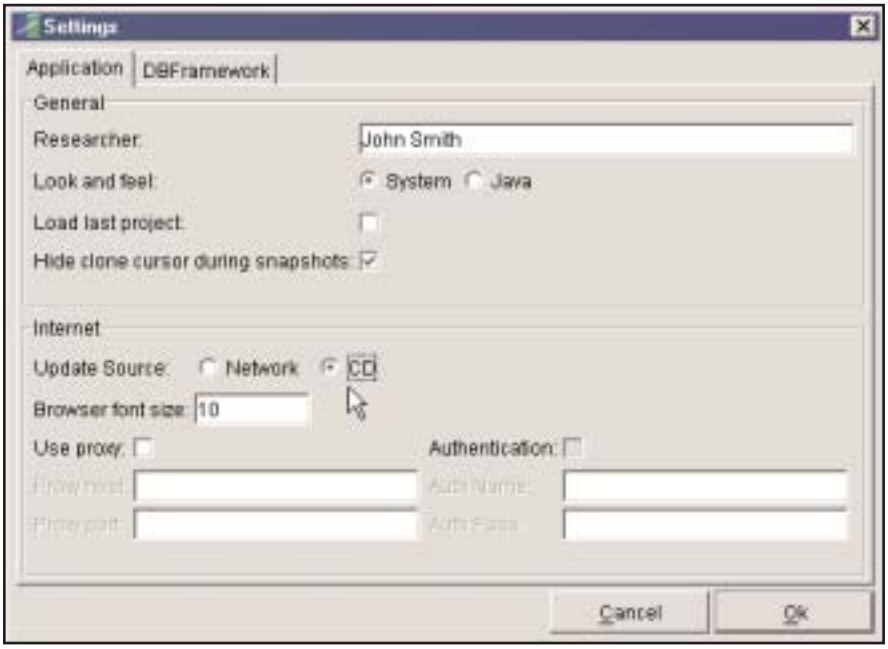

Available Updates
The following Pathways updates are available for download. Please select the updates you wish to download at this time and click "Update" to proceed.
Available Updates
Description
Current
Available
Rat GeneFilters Microarrays Release 3 (GF302)
Human GeneFilters Microarrays Release VIII (GF207)
Human GeneFilters Microarrays Release IX (GF208)
Human Bone-Specific GeneFilters Microarrays (GF22...
Pathways main program jar
Help Jar
OneWay PlugIn
KMeans Clustering Algorithm
Version 1 (Apr 24, 2001)
original version of feature Rat GeneFilters Microarrays Release 3 (GF302)
Update
Update All
Cancel

Select items to update, and click *Update*, or click *Update All*. When a large number of updates are available, download times are reduced by selecting a single update, rather than all of the updates.

When the updates are complete, a dialog box appears, and it indicates the status of the updated files. **Save the project, and restart Pathways™ to use the updated data and plug-ins**.

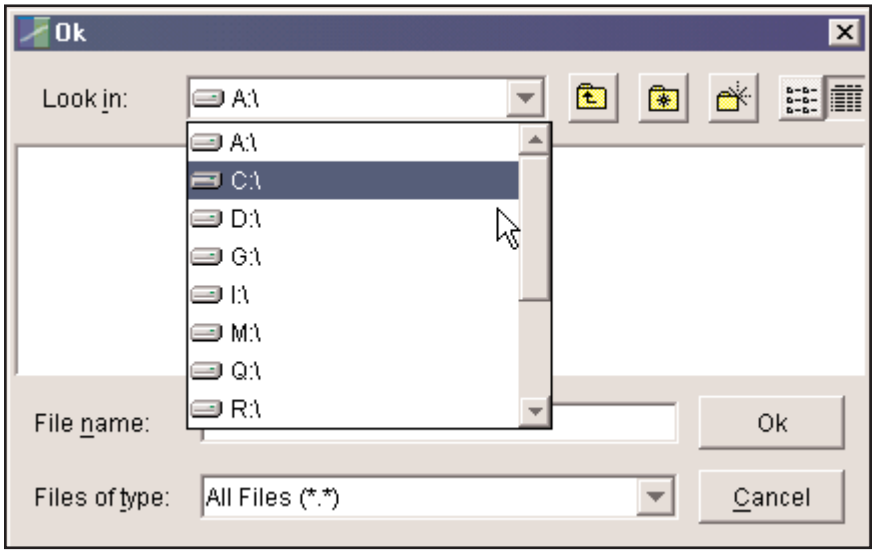
14.5 Updating from CD

For those customers whose Pathways™ installation computer does not have access to the internet, a CD based updating facility is provided. The CD Updater will update both GeneFilters® and program files, giving the user access to the latest GeneFilters® data as well as new program features and bug fixes. Enabling CD updates is accomplished by selecting the **Edit→Settings** menu item. As shown below, there are two updating protocols that are available to the user: **Network** and **CD**. Network updates are the initial default. To change this setting, simply select the CD option and Pathways™ will attempt to update from a CD when the update tool is invoked. CD updates are recommended only for those with internet access problems.

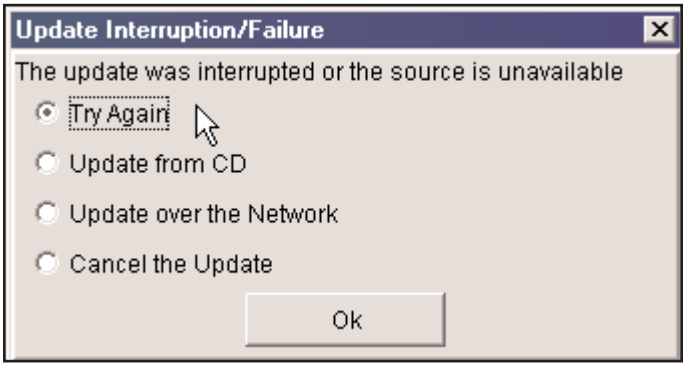


After setting the update source to CD, CD updates may be accessed from the **Tools→Update Pathways** menu item. When the user selects **Tools→Update Pathways**, a file dialog will be displayed. Select the CD drive that contains the Pathways™ Update CD by single clicking on the appropriate drive letter as shown below. Click *Ok* to start the update process.

After the CD update has started, the dialog for selecting and updating data files and programs files is identical to that of the Network updater.



When either a network update (bad connection, update server is down, *et cetera*.) or a CD update (unreadable disk, incorrect cd location) fails, Pathways™ will display an **Update Interruption/Failure** dialog as shown below. This dialog contains four choices. The user may retry the update, change the update source to CD, change the update source to network or the update can be cancelled. If the user elects to retry the update, Pathways™ either tries to re-establish the network connection or presents the user with a file dialog box, depending on the update source. The second and third choices temporarily change the update source and try to update from that new source. This change is only temporary and does not affect the update source as defined in the settings dialog box. Finally, if cancel is selected the update process is aborted, and the user is returned to the Pathways™ main window.



Chapter 15: Examples

15.1 Introduction to Example 1

This section is a quick reference for a basic application using the Pathways™ framework: comparison of two microarrays to analyze the intensity ratios and differences. Parts of the process, such as importing, have been explained in detail in previous chapters. The aim of this example is to provide a guide for a simple project from start to finish. GeneFilters® microarray images are used in this example.

In this example, there are three steps in the Pathways™ Quick Start palette.

1. **Import new microarray images.**
2. **Use the project wizard to create a new project.**
3. **Compare two microarray images and print a report.**

Interactive importing of two GeneFilters® microarray images in the template mode are outlined in this example. Finally, printing a report of the project is demonstrated.

The Pathways™ Quick-Start palette appears at start-up, unless it is disabled by checking the **Do not show at start-up** box at the bottom of the palette. Alternatively, the Quick-Start palette can be selected from the Tools main menu and clicking on Quick-Start or pressing Ctrl + K.

15.2 Example 1 Step One: Import Microarray Images

From the Quick-Start palette, select the Import microarray icon.

When the Import dialog box appears, perform the following steps.

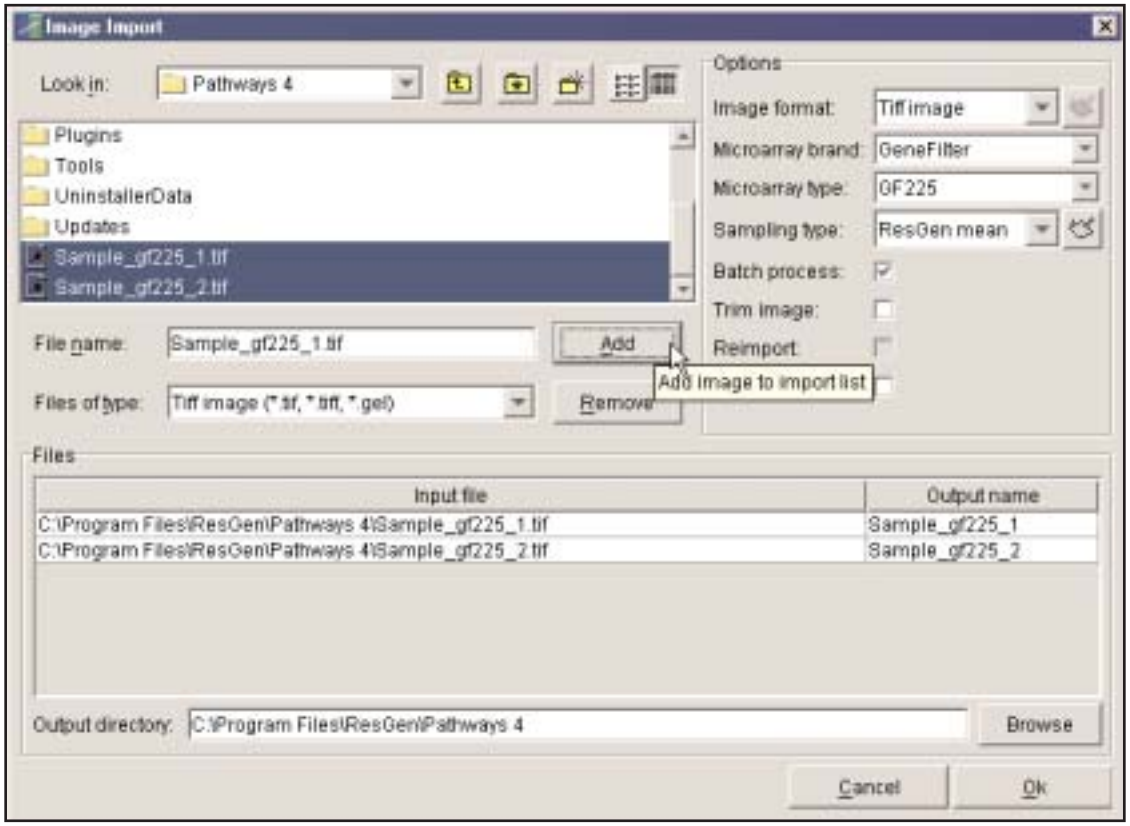
1. **Select the images to import from the appropriate directory / folder / file from the 'Look in' field.**

2. **Complete or select fields in the import dialog box to specify microarray name and brand, image format, sampling type, file type, output directory, *et cetera*.**

3. **Click the *Add* button and *Ok* to continue importing.**

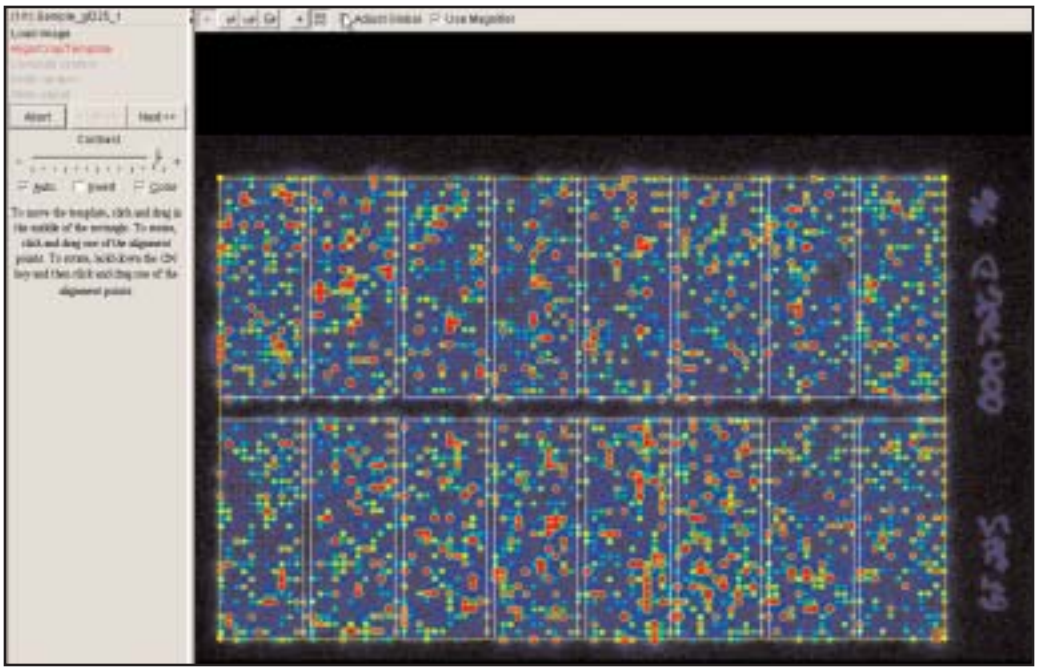
Uncheck the Batch process box for interactive importing.

In this example, Tiff images named Sample_gf225_1.tif and Sample_gf225_2.tif are selected from the Pathways™ 4 folder. The output file is selected by clicking the *Browse* button.

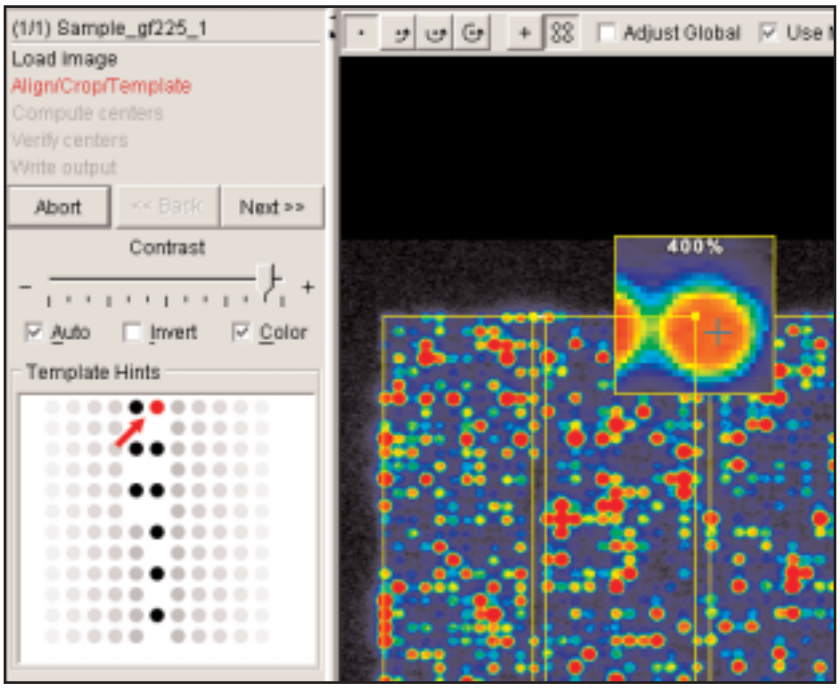


The interactive importing process in this example consists of three steps after the image has been automatically loaded.

1 **Align Template** Click the *Template* button. Instructions on creating, resizing, and moving the template appear on the screen to the left of the image.



Uncheck the *Adjust Global* box, and check *Use Magnifier* to fine tune the alignment using the template hints that appear on the screen to the left of the image. In this example, 16 alignment points are adjusted for the GeneFilters® microarray images.



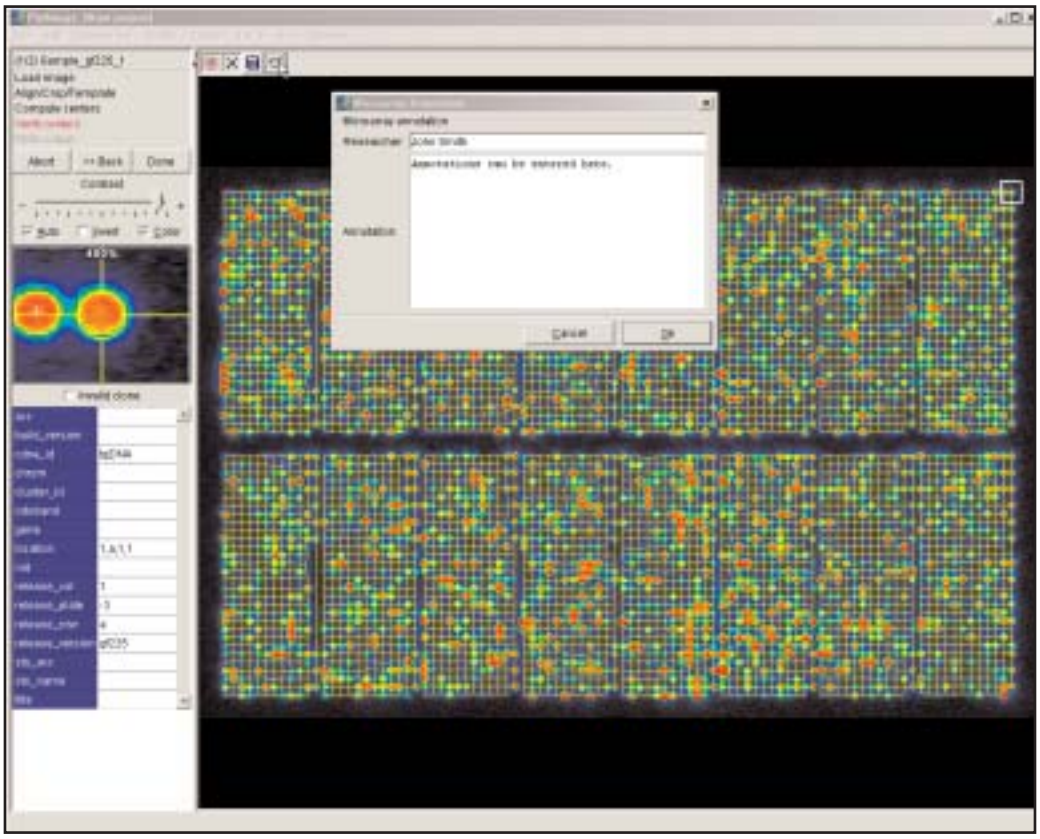
In the magnifier box, the alignment points can be moved by left-clicking anywhere in the magnified image. Up and Down arrows on the keyboard increase or decrease magnification when the magnifier window is active.

2 Compute centers

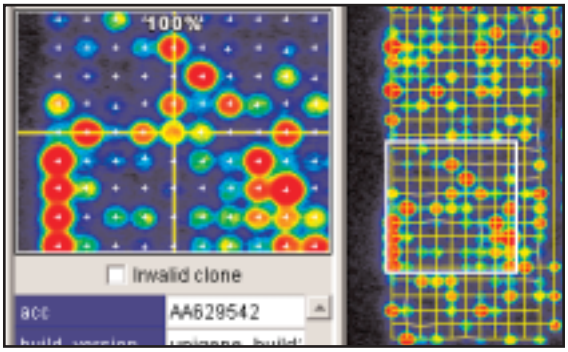
Click the *Next* button after alignment. Centers are computed automatically.

3 Verify centers

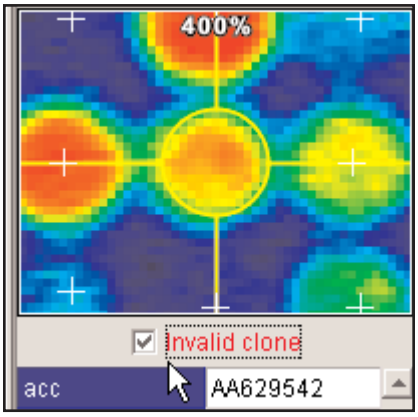
Verify centers by clicking the crosshair on any alignment point in the image. A detail viewer in the window to the left shows the detail view of each point. From this window, spots can be manually centered.



To add the research name and notes on the project click the **Annotation** button on the toolbar. Yield a better picture of the alignment by zooming in and out on the detail viewer with the up / down arrow keys.



To manually invalidate a clone, check the Invalid Clone box in the Detail View area.



Click *Done* if the alignment is satisfactory, or click *Back* to realign the image.

The importing process for the first image is complete. When the next image is loaded automatically, repeat the preceding steps. The importing process is completed, and the Quick-Start palette reappears on the main screen when no more images are selected for importing.

15.3 Example 1 Step Two: Create a Project Using the Project Wizard

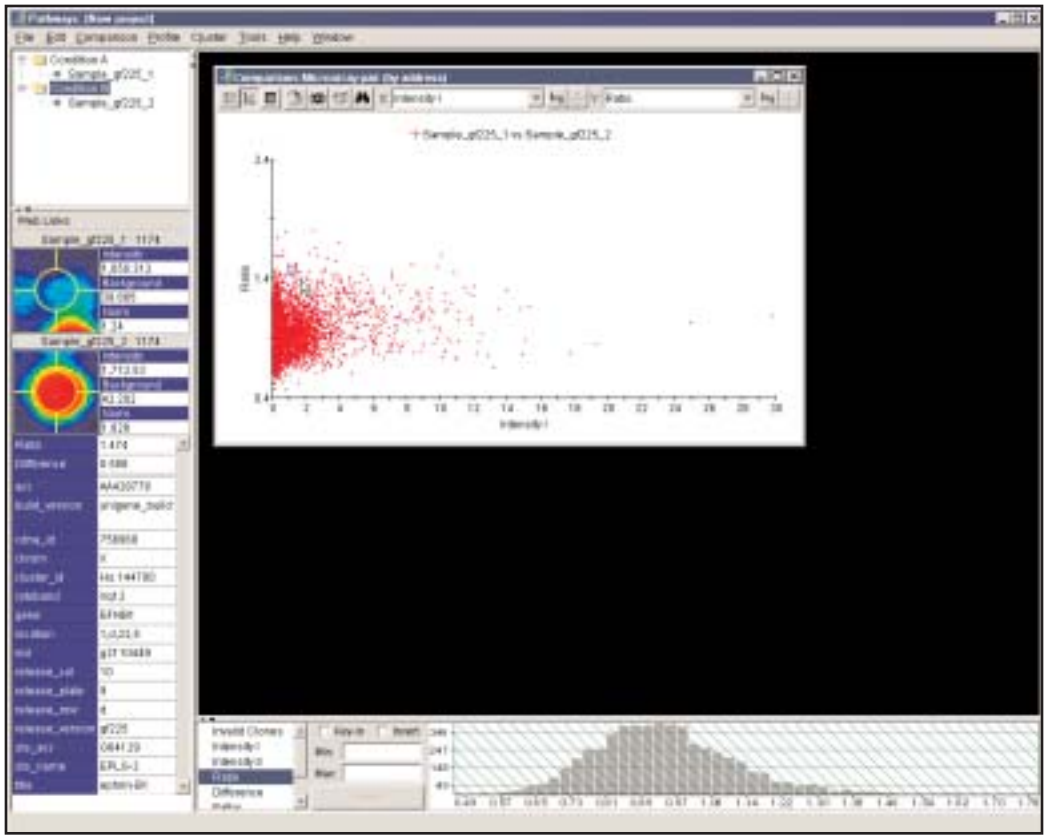
The Project Wizard icon on the Quick-Start opens a wizard that guides project creation. Creating a project allows a researcher to organize the data for analysis, save the data, and reopen the data later. Setting up the project includes specifying the kind of project and choosing the microarray images from a list of imported images. In this example, a project is created to compare the intensity ratios and differences of the two imported images.

To start a new project, perform the following steps.

1. **Click the Project Wizard icon in the Quick-Start palette.**
2. **Select 'Compare intensities of two microarrays' as the project type.**
3. **Enter Project name and Researcher's name.**
4. **Choose the two microarrays.**
5. **Select the normalization type (in this example, Data Point Normalization).**
6. **Click *Finish* to exit the wizard.**

15.4 Example 1 Step Three: Comparison Analysis & Report Generation

After exiting the Project Wizard, the workspace displays the comparison data for analysis. Showing the intensity ratios in a red / green overlay, the synthetic view is the default setting. Data can also be viewed as a scatter plot or table. Plots and tables may be saved as images. In this example, the data appears as a scatter plot (below).



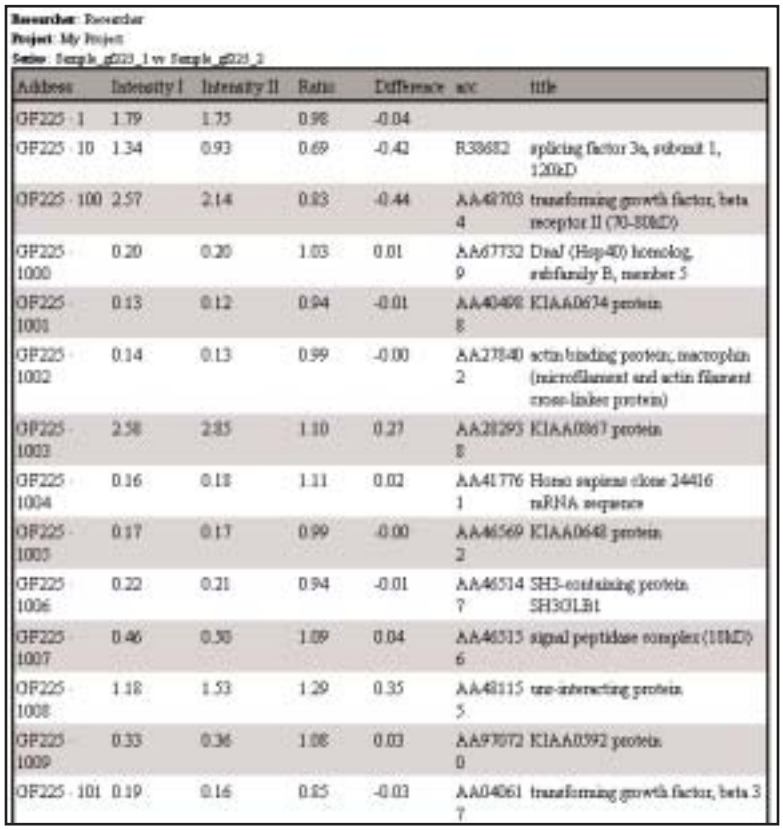
To generate a report, perform the following steps.

1. **Click the *Report* button to generate the report wizard.**

2. **Select how to output the report (*e.g.* printer).**

3. **To include information that appears in the report, check the boxes on the right, and complete the description fields.**

4. **Click the *Print* button to generate a Report Preview window that allows the report to be previewed and printed.**



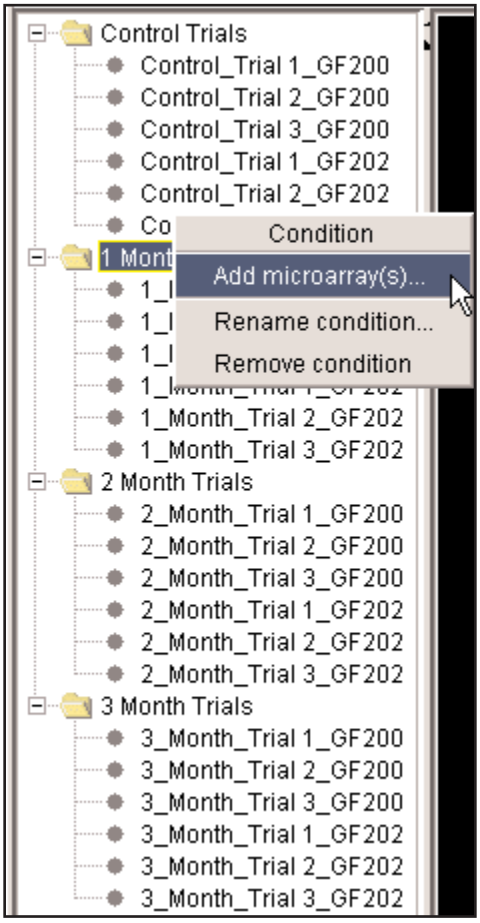
Researcher: Researcher
Project: My Project
Series: Sample_gf225_1 vs Sample_gf225_2

Address	Intensity I	Intensity II	Ratio	Difference	acc	title
GF225 - 1	1.79	1.75	0.98	-0.04		
GF225 - 10	1.34	0.93	0.69	-0.42	R38682	splicing factor 3a, subunit 1, 120kD
GF225 - 100	2.57	2.14	0.83	-0.44	AA48703 4	transforming growth factor, beta receptor II (70-80kD)
GF225 - 1000	0.20	0.20	1.03	0.01	AA67732 9	DnaJ (Hsp40) homolog, subfamily B, member 5
GF225 - 1001	0.13	0.12	0.94	-0.01	AA40498 8	KIAA0674 protein
GF225 - 1002	0.14	0.13	0.99	-0.00	AA27840 2	actin binding protein, macrophin (microfilament and actin filament cross-linker protein)
GF225 - 1003	2.58	2.85	1.10	0.27	AA28293 8	KIAA0867 protein
GF225 - 1004	0.16	0.18	1.11	0.02	AA41776 1	Homo sapiens clone 24416 mRNA sequence
GF225 - 1005	0.17	0.17	0.99	-0.00	AA46569 2	KIAA0648 protein
GF225 - 1006	0.22	0.21	0.94	-0.01	AA46514 7	SH3-containing protein SH3GLB1
GF225 - 1007	0.46	0.50	1.09	0.04	AA46515 6	signal peptidase complex (18kD)
GF225 - 1008	1.18	1.53	1.29	0.35	AA48115 5	ure-interacting protein
GF225 - 1009	0.33	0.36	1.08	0.03	AA97072 0	KIAA0592 protein
GF225 - 101	0.19	0.16	0.85	-0.03	AA04061 7	transforming growth factor, beta 3

15.5 Example 2: Complex Time Study

The following example describes the use of Pathways™ for a more complex project: a time study with repeat data. The example offers an overview of the analysis capabilities of Pathways™, and there is a brief description on the creation of the project and the analysis windows.

First, an Empty Project is created from the new project wizard. Conditions and microarrays are added using menus accessed by right clicking in the project tree.

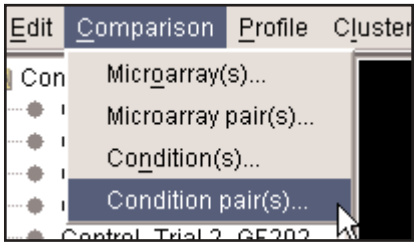


This project shows four conditions in a time study: Control and 1, 2, and 3 Month trials. Each condition has two microarray types: GF200 and GF202. Each microarray is repeated in triplicate for each condition. Repeated elements allow statistical data analysis.

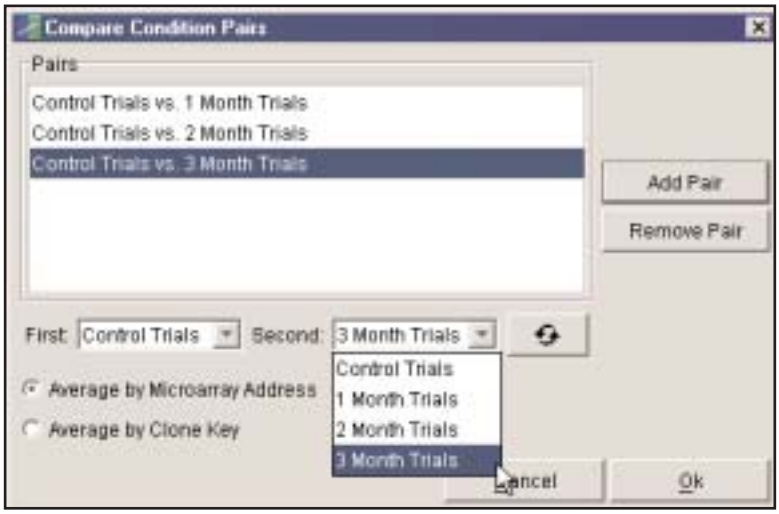
15.6 Example 2: Comparison Analysis

Comparison analysis obtain a broad overview of the data by displaying data from entire microarrays or conditions in a single plotting window with different series overlaid or with multiple table separated synthetic microarray or table windows.

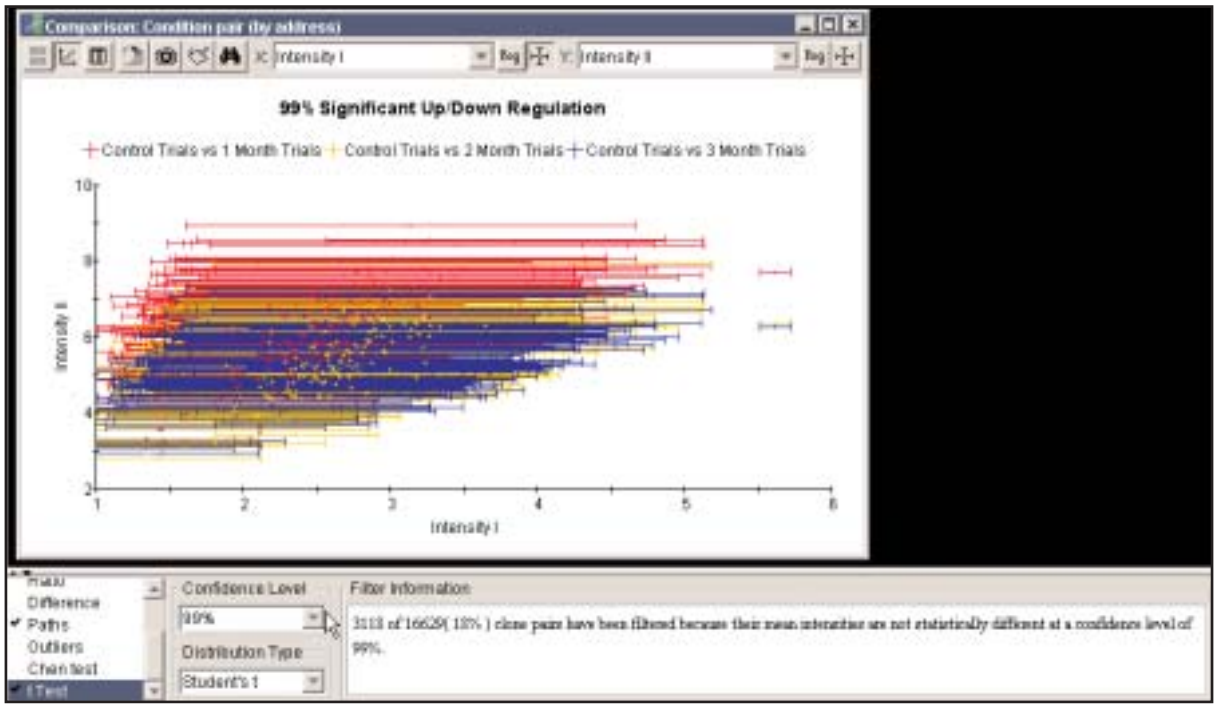
For the current study, clones that are up or down regulated are determined across the time study in comparison with the Control condition. A comparison view is first created by selecting **Compare→Condition Pairs** from the main menu.



Conditions are used for this analysis, rather than microarrays, because both microarray types (including repeats) will be reviewed across all conditions. A condition pair is chosen to observe the upregulation and downregulation of the clones, relative to the control condition. Therefore, pair each condition in the time study with the control condition to establish differential expression.



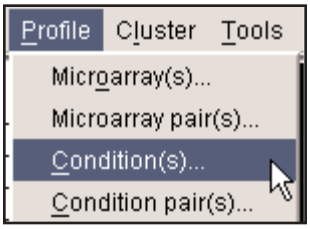
After selecting the condition pairs, click the *Ok* button to generate the condition analysis window. The first view shown is the scatter plot. Specify a 99 % confidence interval for the t-Test filter (the points shown have a 99 % likelihood of being differentially expressed) to ensure a statistically significant difference in expression.

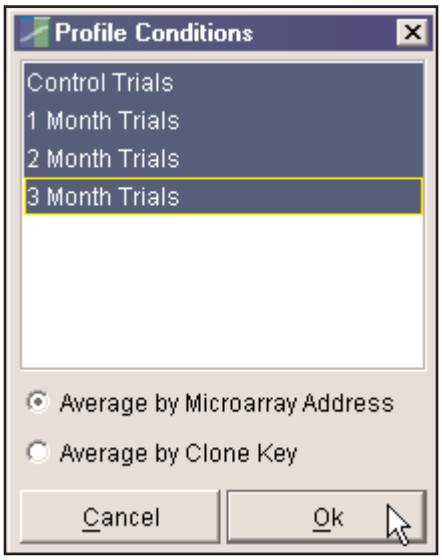


A title has been added to the plot, and error bars are enabled (these options are established in the chart customization dialog box, which is generated by right clicking on the chart).

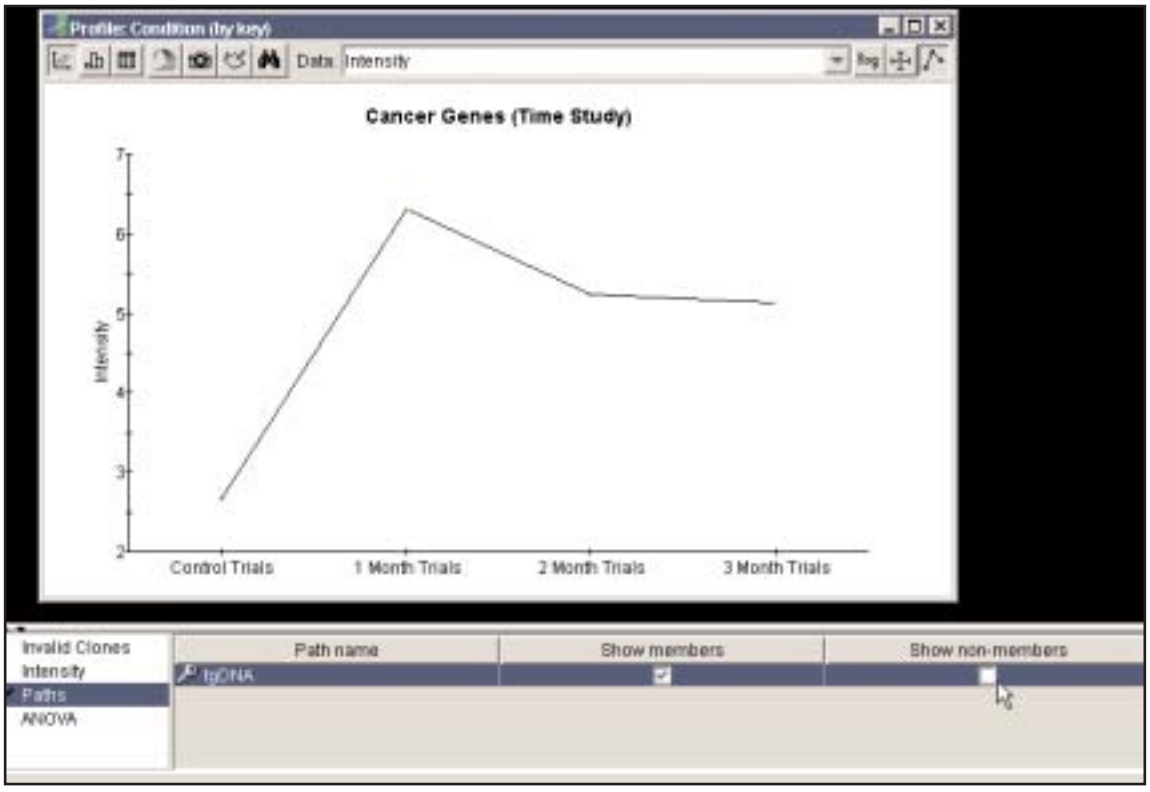
15.7 Example 2: Profiling Analysis

After reviewing the differential expression for the experiment, focus on how the genes that are associated with cancer behaved in the study. To analyze expression levels, rather than differential expression, choose the **Condition(s)** grouping for the analysis from the **Profile** menu.

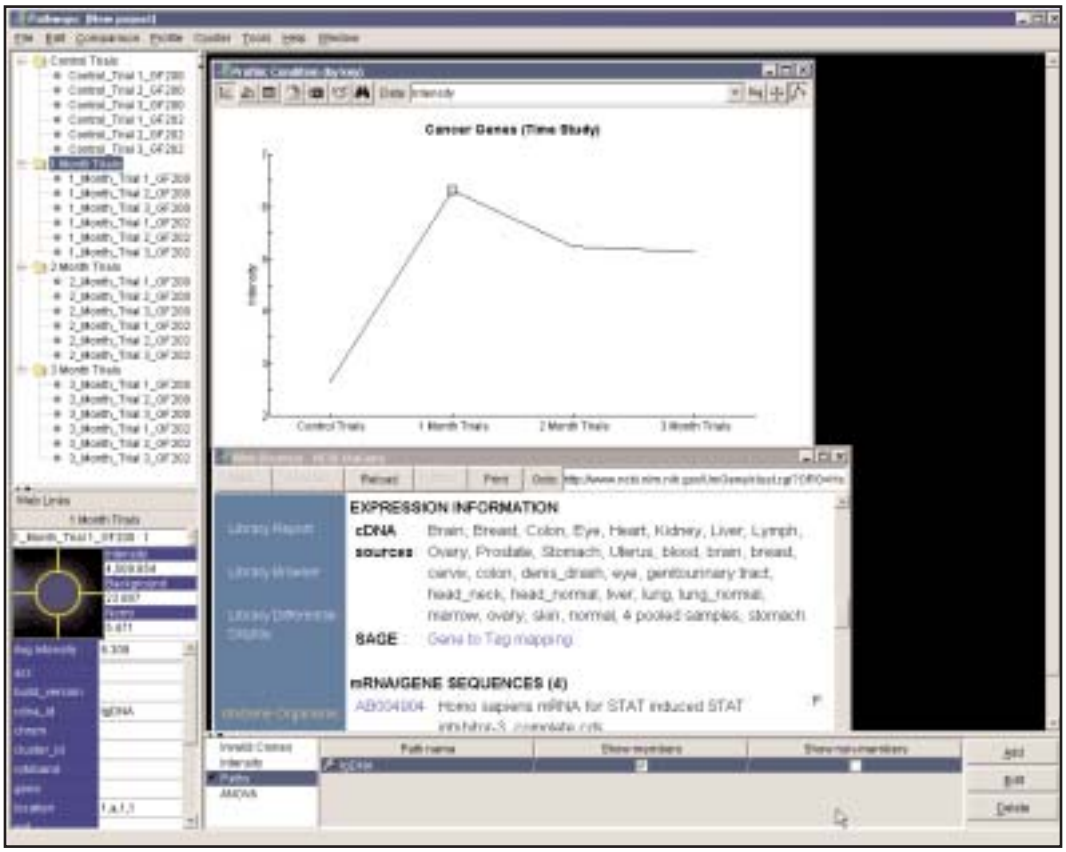




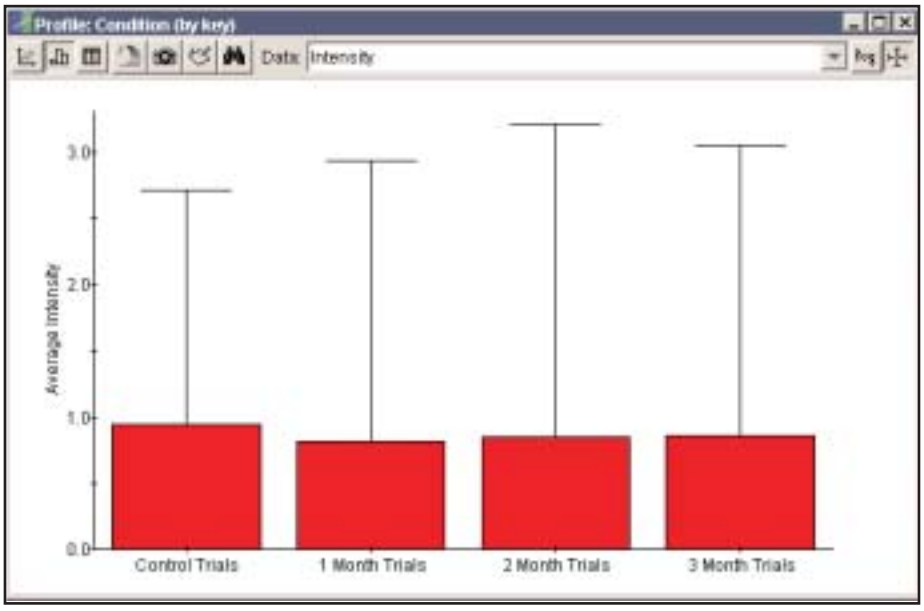
Isolate the cancer genes by unclicking *Show non-members* in the Path filter list. The chart title is entered in the chart customization dialog box, which is generated by right clicking.



To learn more about the most highly expressed gene in the control study, select this gene from the chart view, and activate a web link to Unigene.

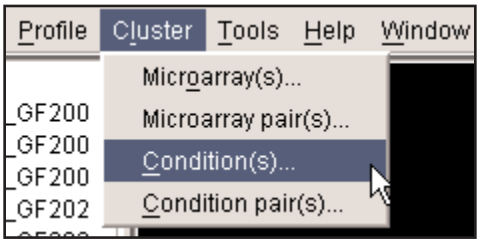


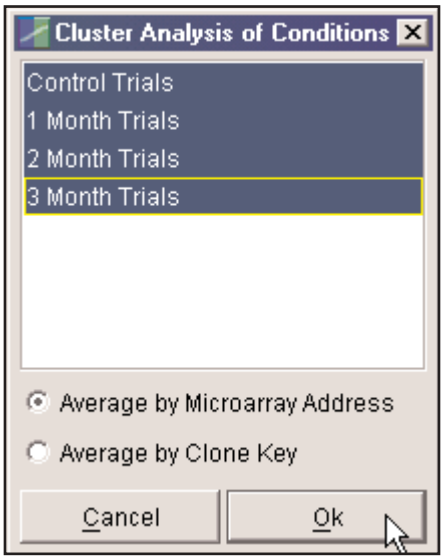
Finally, to view the overall trending of the cancer genes, rather than the detailed plot of each gene, open the bar chart view. The bar chart view presents the average of the cancer genes, along with standard deviation bars.



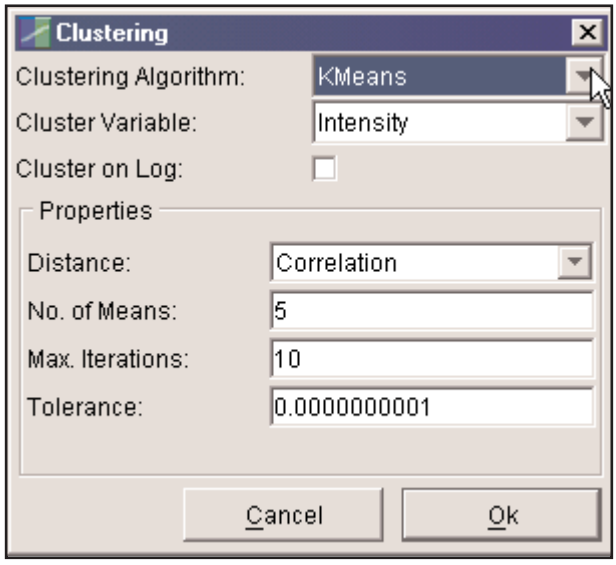
15.8 Example 2: Clustering Analysis

As a final step in the time study analysis, locate clones that behaved similarly to the cancer genes. Clustering the analysis data allows us to group the set of clones into clusters of similarly behaving clones. As with the profiling analysis, start with the appropriate menu selections for a condition grouping.

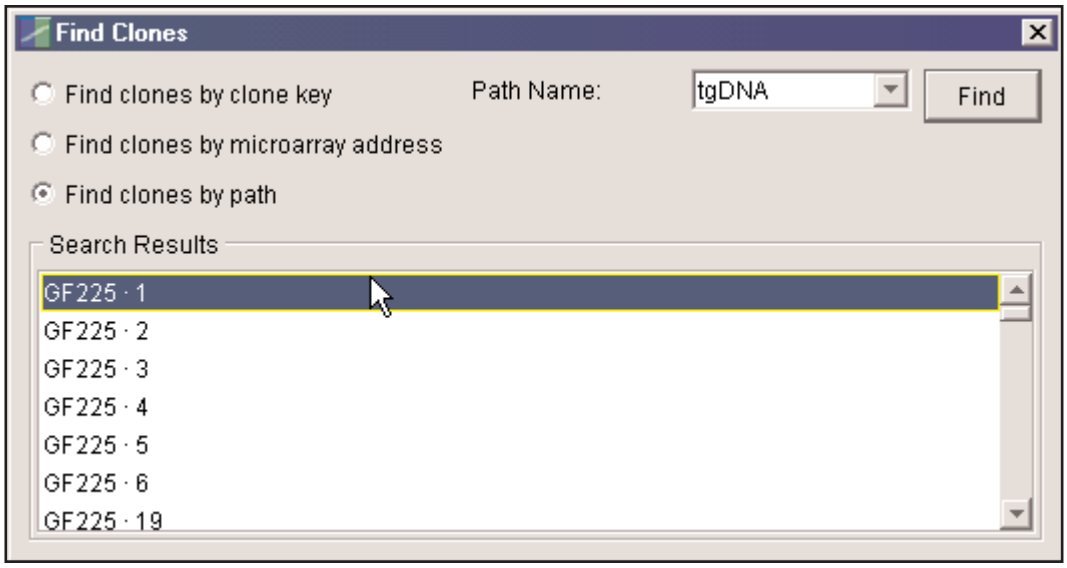




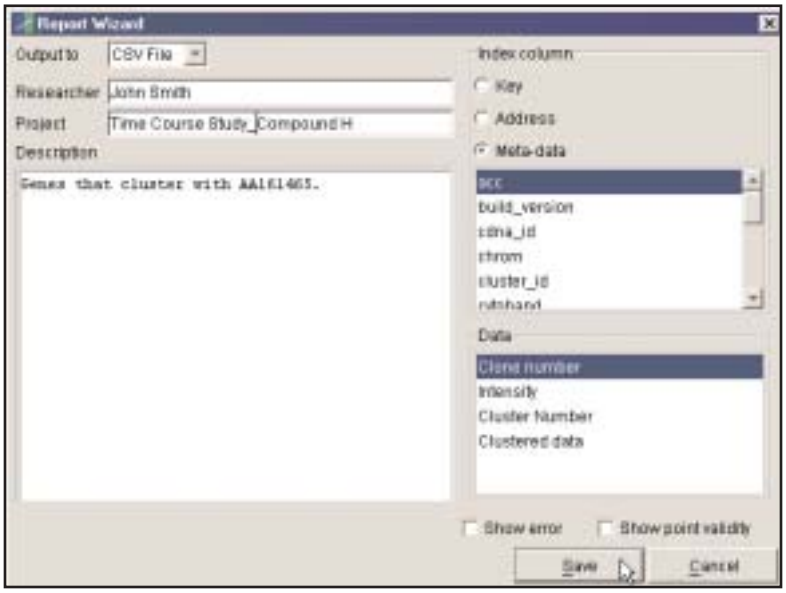
The **Clustering** dialog box allows us to specify the type of clustering algorithm and the properties for the algorithm. Choose a KMeans cluster with 50 means (clusters).



To view the clusters for specific cancer genes, activate the cluster filter at the bottom of the screen. Open the **Find Clones** window from the cluster window toolbar, and click on the clones in this path to display the corresponding clusters.



Finally, save the results from the study to a text file for later reference by opening the **Report** dialog box from the cluster window toolbar.



Book VI: Appendices

Appendix I: ResGen™ GeneFilters® Microarrays

I.1 Introduction

Being able to profile gene expression patterns of tens of thousands of genes in a single experiment, cDNA microarrays have generated great interest in the past few years. When combined with the Pathways™ 4 software package, ResGen™ GeneFilters® microarrays offer the scientific community an opportunity for low cost entry into the microarray arena without compromising experimental quality. By offering a reusable microarray system without the need for complex and expensive laboratory setup, this powerful tool is affordable to every laboratory involved in genomics research.

This Appendix includes an overview of ResGen™ GeneFilters® microarrays' technology, as well as brief descriptions of each microarray product: GeneFilters® Mammalian microarrays (human, rat, and mouse); GeneFilters® Yeast microarrays and MyArray™ DNA. The many different applications of microarray technology, such as differential gene expression, gene discovery, genotyping, pharmacogenetics, *et cetera*, can greatly accelerate genomic research.

I.2 The GeneFilters® Microarray System

Templates for genes are obtained from ResGen™ cDNA libraries arrayed in multi-well culture plates. The insert DNAs are prepared and quality-control checked before being printed onto positively charged nylon membranes using an automated robotic system. The DNA is then UV-cross linked to the membranes. These printed membranes are the ResGen™ GeneFilters® microarrays.

In a typical experiment (Fig. 1), total RNA from both control and experimental samples are reverse transcribed and simultaneously radioactively labeled by incorporation of α-^{33}P dCTP in the reverse transcription reactions. These labeled probes are then purified and used in parallel hybridization experiments. Expression levels of the genes on the arrays are observed as hybridization "spots." A phosphor imaging system detects the intensity, and the image data set is then imported into Pathways™ 4 for analysis. Pseudo-colored images are created in Pathways™ 4 for each data set. When comparing expression patterns between control and experimental samples, the two pseudo-colored images are merged to show intensity differences and ratios. Information related to each clone, including gene name, clone ID, accession number *et cetera*. are attached to each target. Tools for data analysis and data management are available in Pathways™ 4.

For detailed experimental protocols, visit the http://www.resgen.com/products/GF200_protocol.php3.

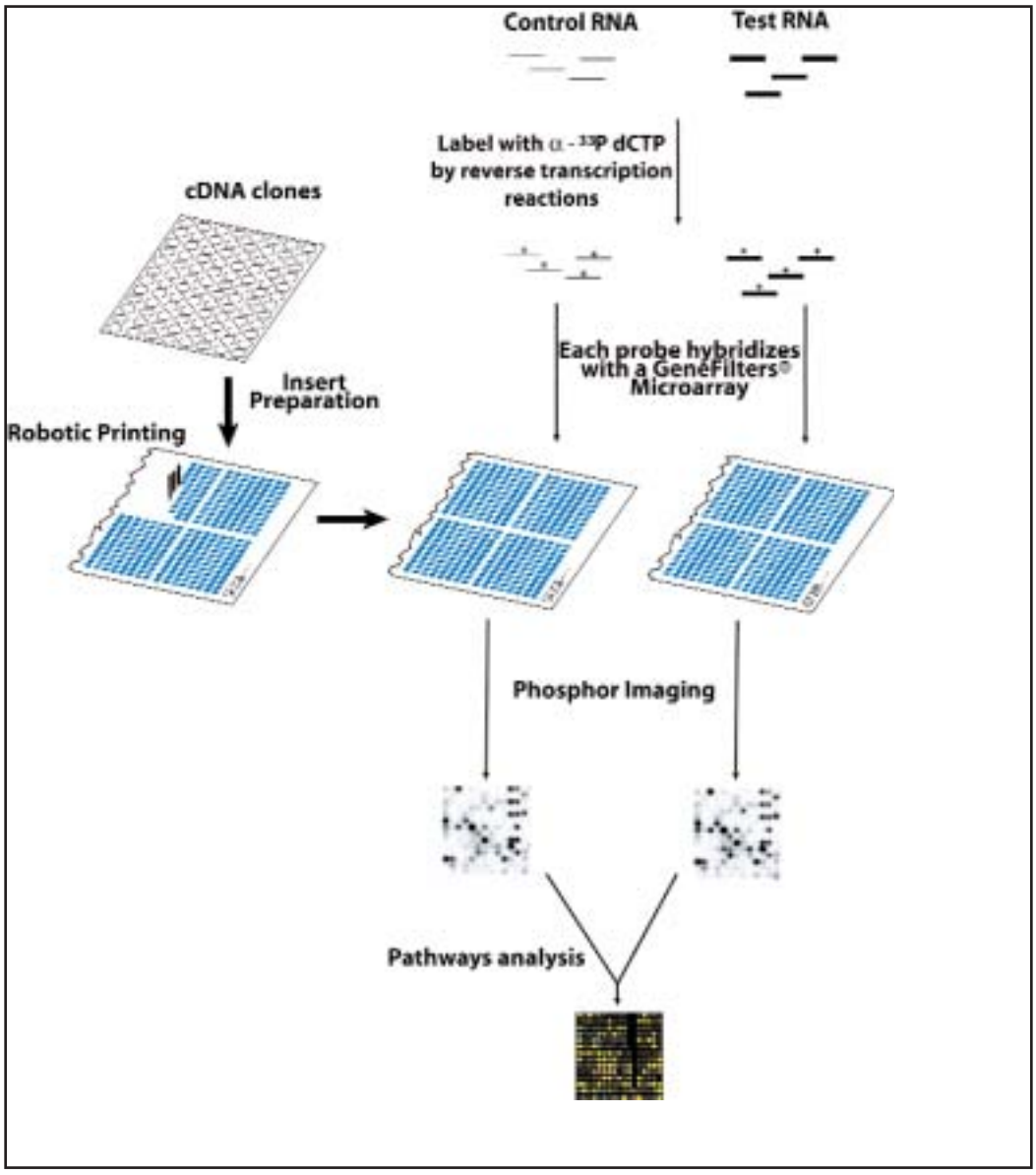


Figure 1. An overview of the GeneFilters® microarrays system

On-line resources for GeneFilters® microarrays

For a current list of GeneFilters® microarray products, visit http://www.resgen.com/products/MammGF.php3 for GeneFilters® Mammalian microarrays, and http://www.resgen.com/products/YeastGF.php3 for GeneFilters® Yeast microarrays.

For a list of genes spotted on each membrane, visit the ResGen™ ftp site at ftp://ftp.resgen.com/pub/genefilters.

For a query for identifying each spot on a membrane, visit http://www.resgen.com/resources/apps/genefilters/.

I.3 Layout of GeneFilters® Microarrays

On the GeneFilters® microarrays membranes, there is a system of controls, including total genomic DNA and putative housekeeping genes. The controls help to orient and align the membrane when using Pathways™ software. Familiarity with GeneFilters® microarrays membrane layout facilitates image importing.

A GeneFilters® Mammalian microarrays

The GeneFilters® Mammalian microarrays contain a total of 5,184 genes spotted on a single nylon membrane. Each membrane is cut in the upper right corner for orientation and the DNA is on the labeled side of the membrane. Figure 2 illustrates the general format of a GeneFilters® Mammalian microarray membrane. Each membrane is divided into two fields. Field 1 is at the top and Field 2 is at the bottom. Each field is then further divided into eight grids. Grids are laid out right to left, A through H in each field and then organized into 12 Columns and 30 Rows. Columns are numbered 1 to 12, right to left in each grid. Rows are numbered 1 to 30, from top to bottom, in all grids, in both fields. Control positives are in Column 1, Rows 1, 3, 5, 7, 9, 11, 25, 27, and 29; and in Column 2, Rows 1, 3, and 5, in each grid. Also in each grid, the housekeeping genes are in Column 1, Rows 13 through 24 (Fig. 2 and Fig. 3). The spacing between each spot is 750 microns from center to center (Fig. 3).

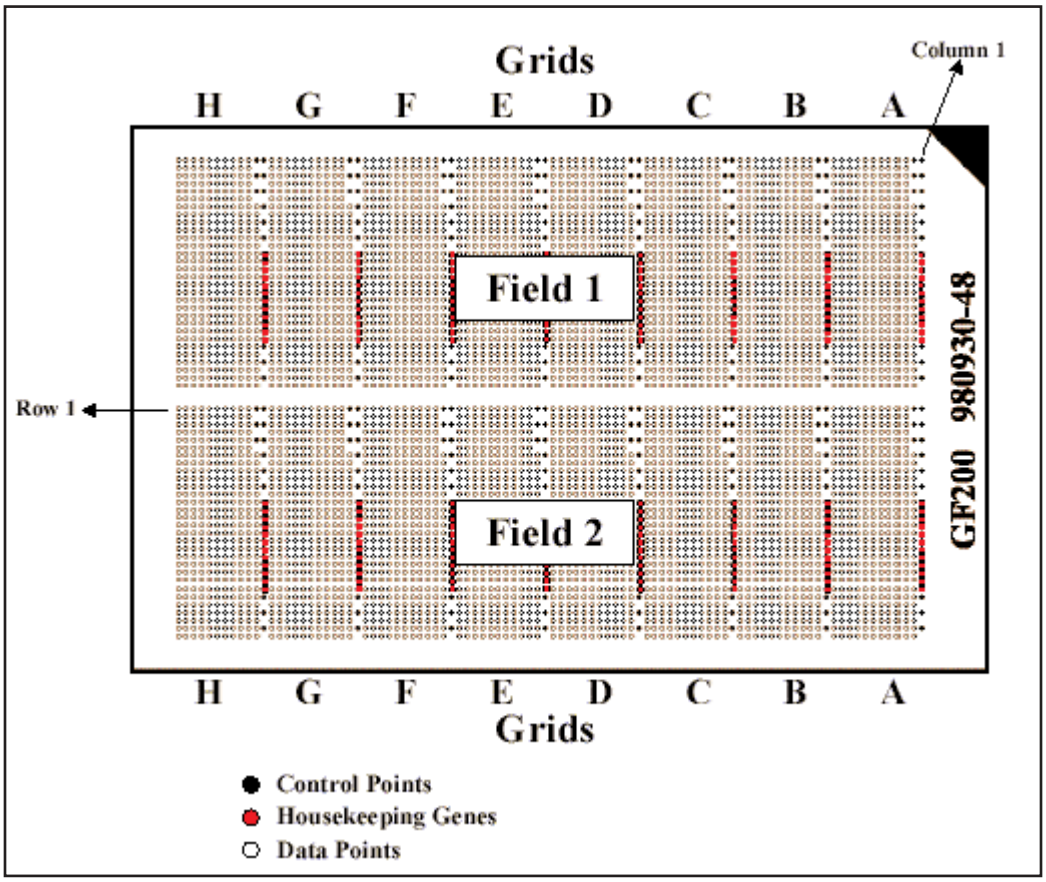


Figure 2. Example of a GeneFilters® Mammalian microarray format (all releases). The 'control positive' or total genomic spots are shown as filled black circles. The putative 'house keeping genes' are shown as filled red circles. Data spots are shown as open circles. GeneFilters® Tissue Specific and Named Gene microarray releases have the same overall format, but there may be some areas on the membranes without DNA spots, depending on the number of cDNAs included on each membrane.

B GeneFilters® yeast microarrays

The GeneFilters® Yeast microarrays consist of 6,144 gene ORFs spotted on a set of two nylon membranes (GF100-I and GF100-II) each containing 3,072 ORFs. Each membrane is cut in the upper right corner and the DNA is on the labeled side of the membrane. Figure 4 illustrates the format of the GeneFilters® Yeast microarrays membranes. Each membrane is divided into two fields; Fields 1 and 2 for GF100-I and Fields 3 and 4 for GF100-II. Fields 1 and 3 are at the top, Fields 2 and 4 are at the bottom. Each field is further divided into eight grids per field. Grids are laid out right to left, A through H in all fields. The grids are organized into nine Columns and 24 Rows. Columns are numbered 1 through 9, right to left in each grid. Rows are numbered 1 through 24, from top to bottom, in each grid. Control positives are in Column 1, every other Row, in all grids, in both fields. The spacing between each spot for GeneFilters® Yeast microarrays is 1,000 microns from center to center.

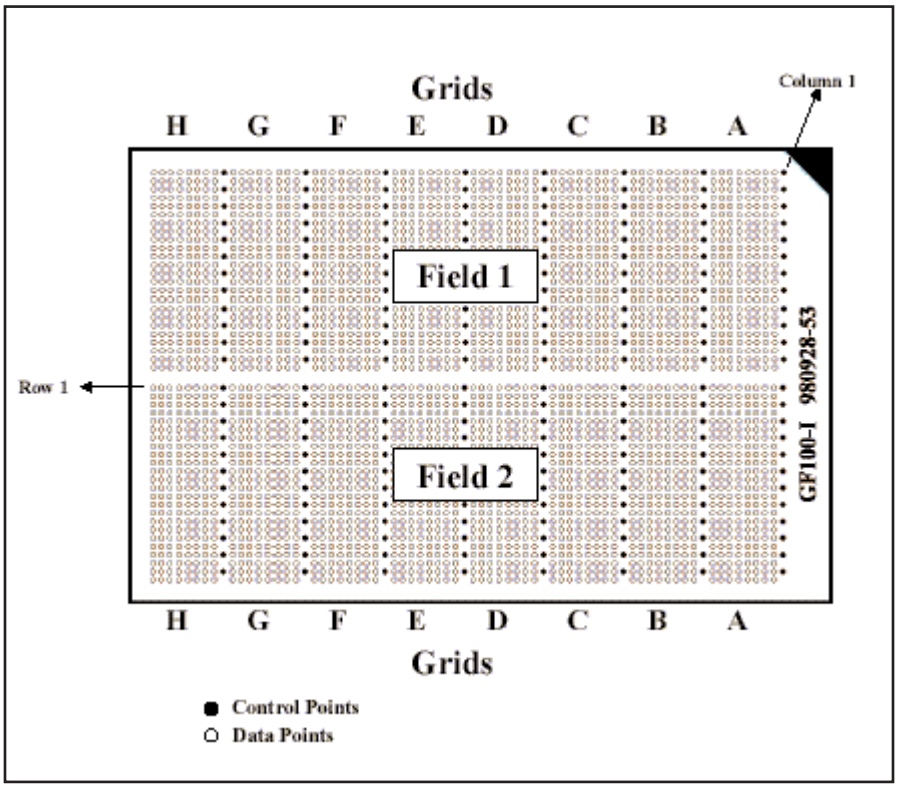


Figure 3. Example of a GeneFilters® Yeast microarray format (all releases). The control positive, or total genomic spots, are shown as filled black circles. Data spots are displayed as open circles.

Appendix II: Migrating to Pathways™ 4 Universal

II.1 Introduction

Compared to Pathways™ 2, Pathways™ 4 offers additional capabilities like statistical analysis tools and graphical visualization of data. Data storage has been improved in Pathways™ 4, with data stored in sharable files.

Other useful features include hyperlinks to online databases and an available update service to the ResGen™ Data Server. Pathways™ 4 has the flexibility to accommodate other commercial or custom microarray formats, rather than ResGen™ GeneFilters® microarrays alone.

This software supports other image formats. Pathways™, Tiff, and Fuji image formats may be imported, and there the software can be extended to include other image formats. From image format and sampling to normalization and data analysis, Pathways™ 4 can be customized. Along with many others, these changes allow Pathways™ 4 to perform as a complete package for the analysis of differential gene expression using microarray data.

II.2 Image Import and Alignment

Image format and microarray format capability

Pathways™ 4 supports multiple image formats including Pathways™, Tiff and Fuji formats. Because different researchers have access to different scanning equipment, other image formats can also be accommodated. Unlike Pathways™ 2.0, Pathways™ 4 can be used for microarray formats other than ResGen™ GeneFilters® microarrays. The software can be customized to analyze microarray products marketed by other vendors or even on custom arrays through the use of the array designer. The analysis tools featured in Pathways™ 4 Universal are made available to researchers no matter what type of microarray they decide to use for their experiments. These features are unavailable in Pathways™ 4 GeneFilters®.

Batch import

Data from Pathways™ 2.0 images must be reimported into Pathways™ 4 using the original images. These images must be generated by a phosphor imager, not by Pathways™ 2.0, a necessary step because of the different way that data is handled and stored in Pathways™ 4. With the 'batch importing' feature, reimporting images is faster, and the process is easier. Batch importing automatically imports multiple images using the software's autocentering algorithms. The images can then be visualized to ensure proper alignment before continuing with analysis. An option to perform interactive importing allows manual image alignment (see below).

Interactive import

The interactive importing of Pathways™ 4 goes beyond what was available in Pathways™ 2.0. For instance, when an image has been imported in an incorrect orientation, Pathways™ 4 allows it to be rotated. A global alignment feature allows a template to be dragged over the image. This template includes alignment points that help locate the corresponding reference spots on the image. A magnifier with zooming capabilities aids in centering the points for better image alignment. Should there be difficulty finding spot centers, a pseudo-color option is available to help with visualization. After aligning the image, Pathways™ 4 detects clones with a centering algorithm, and a verification window appears. Select spots to check for proper alignment and readjust them, if necessary. These features were unavailable in Pathways™ 2.0 (refer to Importing Images).

II.3 Grouping of Data and Complex Analysis

There is a dramatic improvement once images pass the importation steps and move into the data analysis process. Using the terminology 'microarray(s), 'microarray pair(s),' ‘condition(s),' and 'condition pair(s)' is one of the new concepts of Pathways™ 4.

Once researchers become familiar with these new concepts of data grouping, they will value the global and complex analysis options Pathways™ 4 has to offer (refer to the "Core Concepts in Pathways™ Data Analysis: grouping of data" section). In Pathways™ 2.0, single GeneFilters® microarrays are analyzed for intensities by selecting Analyze GeneFilter® from the tool palette. To look at the ratios of normalized data from two GeneFilters®, select Compare GeneFilters®. These same functions can be performed in Pathways™ 4, but the terms that describe them are different. In Pathways™ 4, these analysis tools are found under the Comparison menu. Analyze a single GeneFilters® microarray by selecting Microarray(s). Analyze two GeneFilters® microarrays by selecting Microarray pair(s) from the Comparison pull down menu. In addition, these options are on the Quick Start menu, which is similar to the Pathways™ 2.0 tool palette.

Multiple microarrays can be examined simultaneously. For example, by adding more than one GeneFilters® microarray when selecting 'microarray(s)' from the 'Comparison' menu, it is possible to view the intensities of filters in an overlay. This means that the intensity data of each filter is still viewed separately and no ratios are calculated. In graphical representations of the data, both the intensities of each filter are displayed on the same graph, each with a different color-coding. In the same manner, multiple sets of microarrays can be added into the 'microarray pair(s)' comparison analysis. Pathways™ 4 also has an option for a new, broader level of data analysis, termed a condition. By grouping data under a condition, a researcher can include more than one microarray (*i.e.*, repeated experiments) and even microarrays of different types (*i.e.*, ResGen™ GF200 and GF211), together in one group. The behavior of that set of data versus that of another set of data (*i.e.* condition A versus condition B) can be analyzed by comparing Condition pair(s). Other Pathways™ 4 analysis tools are profiling and clustering.

II.4 Normalization and Data Filters

Because of the large amount of data generated by microarray experiments, normalize and filter data. Select the way that Pathways™ 4 normalizes data from a preset list, or even customize the process. The software extracts the information to display intensities and calculate ratios from the normalized data.

In Pathways™ 2.0, intensity ratios are displayed in a + / - fashion to show the upregulation and downregulation of genes, respectively. In Pathways™ 4, users can select how ratios are represented. Pathways™ 4 plots the true ratio (not the '+ / -' convention), but it allows the + / - ratios to be displayed when filtering ratios. As in Pathways™ 2.0, Pathways™ 4 offers the ability to filter large sets of data to certain areas through the following techniques.

- **Manipulating histograms**
- **Designating paths or lists of clones**
- **Keyword or string searches**

II.5 Viewing Data

The filtered data can be viewed in various different graphical representations such as scatter plots, histograms, and clustergrams. Of the different graphical representation offered, each is interactive. A clone can be selected from the graph and a detailed view of that clone from the original image appears. The clone title, accession number, cluster ID, and more are also included. The tables, graphs, and reports generated in Pathways™ 4 can be saved and printed for use in papers, posters, and presentations (refer to "Core Concepts in Data Analysis: Reports").

II.6 Data Management and Updates

Pathways™ 4 does not employ the Microsoft Access Database; therefore, it allows users to share data files. After microarray images are imported into the software, the images and data are cataloged in a library. They are organized and saved by microarray type along with the information pertinent to each microarray. For example, the library displays the original image file, image type, import date, and any experimental annotation associated with the microarray that is highlighted. Select microarrays from this library for analysis. Analysis performed on these microarrays is saved as a project, which can be reopened later. Project organization lets a researcher save work. Once analysis is complete, users can generate printable reports.

Other key features of Pathways™ 4 are hyperlinks and data file updates. Clones can be searched for on public databases such as GenBank® and Unigene through hyperlinks. Users can also add hyperlinks to other web sites. To ensure that the data for each clone are current, ResGen™ offers a subscription service to help keep information up-to-date.

II.7 Making the Change

The easiest way to convert to Pathways™ 4 from Pathways™ 2.0 is to use this manual. The Pathways™ 4 manual contains detailed explanations and descriptions of the features contained in the Pathways™ 4 software. Book V of this manual contains a chapter of examples to guide researchers through the basic steps of image importing and analysis. This chapter illustrates how to simulate the functions of Pathways™ 2.0, and it shows detailed examples of how to use the new analysis features of Pathways™ 4. Questions about the Pathways™ 4 software can be answered by emailing the GeneFilters® / Pathways™ technical support group at ccu@resgen.com.

II.8 Migrating from Pathways™ 3

Pathways™ 4 is fully compatible with Pathways™ 3 image files and projects, so current Pathways™ 3 users should encounter no difficulties when they migrate to Pathways™ 4. Changes to the user interface have been kept to a minimum in order to provide the user with a familiar experience.

Pathways™ 4 contains many new features, making the use of Pathways™ 4 files impossible on older versions of the software. Pathways™ 3 users should carefully read through this manual to learn how to access the new features available to them with Pathways™ 4.

Pathways™ 3 users need to obtain a new license key to use Pathways™ 4 from the ResGen™ Customer Care Unit (ccu@resgen.com). Older license keys will not work with Pathways™ 4.

Appendix III: Exporting Images from Fuji Software

The Pathways™ 4 Fuji plug-in reads the Fuji img/inf file combination, which is output by Fuji Bas scanners. The 'img' (image) file contains raw image bytes, while the 'inf' (information file) contains fundamental image information, such as width, height, encoding parameter, *et cetera*. The following paragraphs describe how to export inf / img formats from the Fuji ImageGage© software.

Obtaining Images in MacOS ImageGauge© 3.3
Obtain images by selecting "File | Export | Fuji Exchange Format..." in the menu. Doing this outputs the two files (the ~.img and the ~.inf), which is in the directory read by the converter. It is possible to select "File | Export |RAW...", but the ~.inf information must be supplied to the Fuji plug-in manually in the properties view (see Locating the ~.inf information below).

Obtaining images in Windows ImageGauge© 3.12
The Windows standard format is already in the two file (~.img and ~.inf) form and so is ready to be read by Fuji to Tiff Converter©. The two files must be in the same directory when opened in the converter.

Obtaining images in other ImageGauge© versions
In "File | Export" search for an option "RAW..." or "Fuji Exchange Format...". If "RAW..." is selected, the ~.inf information must be supplied (see Locating the ~.inf information below). When "Fuji Exchange Format..." is selected, the two files are output (the ~.img and ~.inf files), and both files must be in the same directory to be read by the converter.

Locating the ~.inf information
This information can be directly viewed from the file with the ~.inf extension (just open the file in a text editor). If this view is impossible, the information can be obtained from Image Gauge© under "File | File Info..." The appropriate encoding parameters can be entered in the 'properties' view for the Fuji plug-in in the Pathways™ image import dialog box.

Appendix IV: License Agreement

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Glossary

ANOVA. This is an acronym for Analysis of Variance. ANOVA is a statistical analysis plug-in that compares the variance of the data calculated within conditions to that across conditions. If the variances are not the same, then it is an indication that the means are different.

Array Designer. The Array Designer is a tool available to Pathways™ Universal users. It allows any type of microarray to be imported by creating a layout for associating data with an image of the microarray.

Atlas™ Array Gene List. This is a microarray type developed by Clontech Laboratories, Inc. For more information, see http://www.clontech.com/atlas/index.shtml.

Autocentering. Autocentering algorithms analyze microarray experimental images and determine the location of each clone in an experimental image. The process for locating the clone centers does not require user input and is therefore automatic.

Background. The background display shows the computed background intensity for the image. This display is useful when evaluating low intensity points. Spots with intensities at or near the background average could be noise.

Batch. The batch importing mode uses the autocentering algorithms to automatically import multiple microarray images.

Brightness. Brightness adjusts the viewed intensity of the spots in the SynFilter window. If the spots cannot be seen easily, the brightness is low; it can be increased by using the Brightness Slider. If the spots appear to be the same, the brightness is high; it can be decreased by using the Brightness Slider.

Brightness slider. This tool adjusts the brightness or intensities of the spots in the Synthetic Microarray Windows. Sliding the brightness slider to the right increase the brightness; sliding it to the left decreases the brightness level.

Chen test. This test is a statistical analysis plug-in that determines whether two sampled intensities are different, based on a desired confidence level.

Clone key. A clone key is a unique identification string that is present for each clone in a microarray. The clone key is the accession number when available, but it could be any identifier such as "tgDNA" for total genomic DNA. Repeat spottings in a microarray must have the same clone key to be treated as repeats for statistical analysis. The clone keys are contained in the microarray's description file.

Clone number. The clone number is the index of the clone in the microarray (the microarray address is the filter type plus the clone number, *e. g.*, 'GF200 100'). For GeneFilters®, the clone number is ordered by field, grid, row, and column.

CMT™ Map File. This is a Corning Microarray Technology Map File. A microarray type designed by Corning Incorporated. For more information, see http://www.corning.com/CMT/Products/CMTGeneArray.asp.

Compare two microarrays. This button on the Quick Start Palette initiates a Two Microarray Comparison Project.

Conditions. Conditions represent states of previous imported microarray data in an experiment. Each condition can contain one or more sampled microarrays that do not have to be of the same type (*e. g.*, GeneFilters® GF200 could be grouped with GF201).

Control points. Control points are the "landing lights" or positive controls that are used for orientation and in the Pathways™ alignment process for ResGen™ GeneFilters®.

Cropping rectangle. A cropping rectangle is a rectangular box that is used in the importing process to better identify the location of a microarray in an image.

Difference. In a Microarray Pair or Condition Pair analysis window, difference is the numerical value resulting from the subtraction of the Normalized Intensity of one clone on the first data set from the Normalized Intensity of the same clone on the second data set.

Filter. Pathways™ data filters reduce the amount of microarray data that is displayed in analysis windows or shown in reports. Data filters can restrict the displayed clones to a range of intensities or ratios, and / or to a minimum level of statistical significance, and / or to be members / non-members of selected paths.

Framework. A Framework is a collection of modules that work together to provide a method of importing microarray data from various sources. Frameworks are only available in Pathways™ Universal.

.GEL file. This type of image is a form of .TIF file that is acceptable for Pathways™ analysis. See the .TIF File.

GEML™. This is an XML-based tag set that provides a method for exchanging gene expression data and related annotations. It is used for transmitting data independent of the methodology used to collect that data.

GenBank®. This is an annotated collection of all publicly available DNA sequences located at http://www.ncbi.nlm.nih.gov/Genbank/.

GeneFilters® microarrays. This is a reusable microarray system that can be probed by standard auto-radiographic methods, offering low cost entry into the microarray arena. Developed in conjunction with Pathways™ analysis software, GeneFilters® microarrays simplify gene expression analysis and take advantage of the combination of GeneFilters® membranes and isotopic detection.

GeneFilters® Human microarrays. These microarrays consist of a single membrane containing up to 5,184 non-control spots. The membrane (5 cm x 7 cm) also contains controls: genomic DNA monitors the homogeneity of the hybridization, and a series of housekeeping genes is included for orientation and alignment. Each of the non-control spots on the membrane contains at least 0.5 ng of insert DNA from an Integrated Molecular Analysis of Genomes and their Expression / Lawrence Livermore National Laboratories (I.M.A.G.E. / LLNL) cDNA clone containing the 3' untranslated region end of a gene. These clones were isolated, sequenced, and verified to be correct. Insert DNA was denatured and UV cross-linked to the positively charged membrane.

GeneFilters® Yeast microarrays. These consist of a total of 6,144 gene ORFs (Open Reading Frames) derived from the same SGD, individually amplified and spotted onto two nylon membranes. The amplification reactions use specific primer pairs designed to amplify the entire open reading frame. The primers were generated from unique sequences containing the start codon ATG and termination codon (supplied by M. Cherry at Stanford Genome Center). Therefore, the insert DNA consists of the complete open reading frame including the start and stop codons. A robotic device spots approximately 1 / 10 of a microliter of the denatured insert DNA solution on a positively charged nylon membrane. The DNA is then UV cross-linked to the membrane. A system of positive controls, consisting of total yeast genomic DNA, is printed on each membrane, and it is used for orientation and alignment.

Hierarchical clustering. This is a clustering algorithm that works by finding the two closest clones and then calling this a cluster. It repeats this step until all clones have been assigned to a cluster.

Histogram. A histogram is a graphical representation of the distribution of a data set. Histograms divide a data set from the lowest to highest values into equally sized bins. The number of items in the data set that fall into each bin allows a graphical representation of the distribution of the data set.

Housekeeping genes. Housekeeping genes are genes whose expression is required for normal function of the cell. In general, their expression is consistent, regardless of stimulus.

Import microarray. Selecting Import image from the File menu or clicking this button on the Quick Start Palette begins the process of importing a new microarray image into Pathways™ software.

Importing. Importing is the process of loading an image from a phosphor imaging system file into Pathways™ software. In this process, the image must be loaded into Pathways™, the location of each clone must be determined, and the clone intensities must be sampled. The results of this process are stored in two files: the Pathways™ sample file ('.pws' extension) and the Pathways™ image format file ('.pwf' extension). The results are then imported into Pathways™ database, aligned, and intensity data is recorded into the database.

Intensity. Intensity is the numerical value assigned to the level of expression of a gene or ORF through the import and sampling process. Pathways™ analysis uses normalized intensities, and Pathways™ normalizes raw intensity values before proceeding with analysis.

Inverting an image. Inverting an image reverses the background and foreground colors of an image; that is, if looking at dark spots on a white background, inverting the image allows the researcher to view light spots on a dark background.

Java. Java is a programming language that creates programs that run on multiple operating systems (Windows, Linux, *et cetera*). Java is fully object oriented, and it is ideal for writing applications with plug-ins, graphical user interfaces (GUIs), and internet connectivity.

KMeans clustering. This is a clustering algorithm that groups data into a specified number of clusters by finding the center of the cluster and assigning data to the nearest cluster center in an iterative fashion.

Known genes. This is a microarray that contains only genes with known functions as the data points (Catalog #GF211).

Main menu. At the top of the Pathways™ window, this is the bar that reads Files, Edit, ..., and Help. The Quick Start Palette offers a short cut to items in the main menu.

Meta data. Meta data is the auxiliary data such as accession, cluster ID, title, *et cetera*, that is associated with each clone. Meta data is typically read from a description file for the appropriate microarray.

Microarray address. This is an identifier that associates a clone with a spot on a microarray (*e. g.*, "GF200, Clone 500"). The microarray address identifies the spot by location, whereas the clone key identifies only a clone type on the microarray. For example, there are multiple tgDNA clone keys on ResGen™ microarrays (tgDNA is spotted in multiple locations), but a tgDNA spot has only a single microarray address.

Molecular Dynamics Storm. This is a phosphor imaging system available from Molecular Dynamics.

Normalization. Normalization is compensation for global intensity shifts across multiple microarray experiments. It is possible to make two images similar enough to reasonably compare them by using either the control points, data points, paths, or other methods currently installed as normalization plug-ins.

Normalization groups. Normalization groups assign an explicit normalization technique to one or more groups of microarrays.

Packard Cyclone. This is a phosphor imaging system available from Packard Instruments.

Paths. Paths are bookmarks or shortcuts to certain genes. Paths are specified in one of three ways: (1) by microarray address (location on a microarray type); (2) by clone key (unique name of a clone, usually accession number); (3) as a search string(s) with each clone's auxiliary data (*e. g.*, contains 'cancer' in the clone description).

Phosphor imaging system. This is a high-resolution scanner used with radioactive probes hybridized to ResGen™ GeneFilters® or other microarrays.

Plug-ins. A plug-in is a modular programming unit that adds or modifies key algorithms in the Pathways™ analysis process. Pathways™ supports plug-ins for image formats, microarray type, data sampling, normalization, statistical analysis, and clustering.

Pluggable. A component of Pathways is called Pluggable when a plug-in is associated with the component (*e. g.*, clustering).

+ / - Ratio. This is a convention for displaying the ratio of the normalized intensities of two clones that facilitates the recognition of upregulation and downregulation. The + / - ratio of clone 'A' versus clone 'B' is equal to the normalized intensity of B divided by A if the normalized intensity of B is greater than A and otherwise the + / - ratio is negative A divided by B.

Project. Pathways™ projects organize previously imported microarray data into conditions that represent experimental states.

.PWF file. This is an extension for a Pathways™ image file that contains calculated clone locations on the experimental image as well as a full resolution copy of the original experimental image (this copy may be cropped and / or rotated, depending on the alignment of the original image).

.PWS file. This is an extension for a Pathways™ sample file that contains sampled data from a Pathways™ import session. This file contains the data that is used throughout the Pathways™ analysis process.

Quick start palette. This is the picture menu that reappears when a task (such as importing, analysis, comparison, *et cetera*) has been completed or exited. This palette can be enabled or disabled in the Options.

Ratios. Ratios are the numerical value of the Normalized Intensity of a spot on a microarray in a microarray pair or condition pair divided by the Normalized Intensity of the same spot on a second microarray.

Release plate / row / column. These describe the location of the verified clone in the ResGen™ cDNA libraries.

Reimport. Pathways™ image files can be read back into Pathways™ (reimported) without the need for finding the location of the clones. Reimport allows changing sampling technique for a file without dealing with finding clone centers or to adjust clone centers (if the center is found to be in error) without having to deal with global alignments.

Reports. Reports are printable documents compiled by Pathways™ software that allows the data specified in an analysis window to be viewed on-screen, printed out, or saved to a text file.

Rosetta GEML Conductor™. This is a program that converts gene expression data files into GEML™. For more information, see http://www.geml.org/conductor/conductor_home.htm.

Sample image. These are images supplied with Pathways™ software that allow the researcher to practice using the applications.

Sampling. Sampling is the process by which raw image data generate an intensity value for each clone on a microarray. A typical sampling algorithm averages the image data in a circular area of a clone center, yielding a clone intensity value.

Settings. From the Edit menu, this selection allows the researcher to customize some of the applications in the software.

SOM clustering. This is the self-organizing map clustering algorithm. A clustering algorithm that finds clusters in an input data set by mapping the data onto a two-dimensional array of nodes and then running these nodes through a series of iterative calculations.

Synthetic microarray. This is the view of the microarray image(s) currently being analyzed in Pathways™ software. This image is a synthetic, or cleaned-up, version. The spots appear to be the same size (with differing intensities), allowing analysis and comparison between different microarrays.

t-Test. This is a statistical analysis plug-in that determines whether two sampled intensities are different, based on a desired confidence level. The t-Test is a specific application of ANOVA for two conditions.

Template. A template is a computer-generated sketch of the critical points in a microarray that can be overlaid on top of an image. Templates can be stretched and rotated, and template points can be dragged to aid the location of clones in a microarray image during an import process.

Thumbnail. A thumbnail is an enlarged and enhanced picture of a spot, as it appears on the original image stored in the Pathways™ image format file for the current microarray. This picture is intended to be a visual reference, and it is useful only when numerical intensity values are used simultaneously.

.TIF file (or TIFF File). This image file format created by phosphor imaging systems can be analyzed by Pathways™ software.

Unigene. This is a system for partitioning GenBank® sequences into a non-redundant set of gene-oriented clusters, containing sequences representing a unique gene and its associated meta data. It is located at http://www.ncbi.nlm.nih.gov/Unigene/.

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